The Role of Teneurin C-Terminal Associated Peptide (TCAP)-1 in the Regulation of Stress-Related Behaviours

by

Laura Alexandra Tan

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell and Systems Biology
University of Toronto

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2011

Abstract

The teneurin C-terminal associated peptides (TCAPs) are a newly-elucidated family of four bioactive peptides that were found during a screen for novel corticotropin-releasing factor (CRF)-like peptide families. The predicted peptide sequences have the characteristics of a bioactive peptide and are 40 or 41 amino acid residues long. One of the peptides in the family, TCAP-1, has numerous in vitro effects, where it modulates cAMP accumulation, neuronal proliferation, neurite outgrowth, brain-derived neurotrophic factor levels, and possesses neuroprotective effects under alkalotic or hypoxic conditions. However, little is known about TCAP-1’s in vivo effects. Given the structural similarity of TCAP-1 to the CRF family, it is expected that these peptide systems may interact in vivo. The aims of this research were to 1) investigate the role of TCAP-1 on CRF- and stress-induced behaviours in rats, and determine if intracerebral TCAP-1 could modulate stress-induced anxiety-like behaviours; 2) determine the areas of the brain where TCAP-1 is taken up and is active; and 3) investigate the role of TCAP-1’s cytoskeletal modulation on stress-sensitive areas of the brain so as to determine a mechanism for long-term behavioural changes in the brain. I have established that TCAP-1 modulates anxiety-like behaviour in exploratory tests of anxiety, and that TCAP-1 is particularly active in the limbic system, including the hippocampus, amygdala, septum, and medial prefrontal cortex,
and that TCAP-1 increases the dendritic spine density in the hippocampus, a brain area important for anxiety, learning, and memory. These studies have confirmed that TCAP-1 indeed plays a role in stress-like behaviours and modulates stress-related processes in the brain.
Acknowledgments

A doctorate is no doubt a long and arduous process and an adventure that cannot be completed alone. I have been blessed by the best network of supervisors, advisors, coworkers, friends, and family who have supported me throughout my studies at the University of Toronto and have made my time here memorable beyond all imagining.

I have been extremely fortunate to have been supported by a number of sources, including the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of Toronto, and the Department of Cell and Systems Biology. I would not have been able to complete these studies without this support.

As graduate students consider themselves lucky if they have a good working relationship with their supervisor, I consider myself doubly blessed to have been under the wings of Drs. Susan Rotzinger and David Lovejoy. My heartfelt thanks to Susan for her patience and guidance, especially in the early years of my Ph.D. when projects were unspeakably long and data was fleeting. Susan, you have been a stellar role model and I have thoroughly enjoyed our long discussions on science, family, and life. To David, thank you for the stellar projects that have opened up so many opportunities for me. You have put unwavering faith in my abilities over the years and have allowed me to blossom under your supervision. Our conversations, whether about the intricacies of behavioural data or the subtleties of a recently reviewed red wine, have always been stimulating and appreciated. To Susan and David, being your graduate student has been, without a doubt, one of the greatest honours I could have received.

I would also like to thank my wonderful supporting cast of advisors, both official and unofficial. To my supervisory committee members, Drs. Franco Vaccarino and Les Buck, thank you for the many helpful suggestions over the years that have shaped my studies and given me perspectives from two very different points of view. I would also like to thank Dr. Jackson Bittencourt, whose advice through several visits has both challenged and encouraged me in my studies. Special thanks to Ian Buglass, who has helped me navigate the administrative ins and outs of the department.

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undergraduate and an early graduate student; to Gina Trubiani, whose drive has always been an inspiration; Dr. Cláudio Casatti, whose Brazilian hospitality knew no bounds; to Tiffany Ng, for being an incredible friend in the lab, the best of travel buddies, and a lifelong adopted sister; to Dhan Chand, for the many discussions, crazy times on various conference trips, and for pushing the TCAP project along at an dizzying pace; to Tanya Nock, for her infectious smile and ever-needed optimism; to Reuben DeAlmeida and Mei Xu, for their enthusiasm and random tea-times; to Lifang Song for her intense professionalism; and to Rasha Ahmed and Yvonne Kwok, two undergraduate students whose presence in the lab made summers a blast. Lastly, I would like to thank Karen Xu, who worked side-by-side with me through various behavioural projects and who never turned down a request for her exceptional technical wizardry.

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Finally, I am greatly indebted to my wonderful parents, Rose and Michael, who have always encouraged me to follow the career that made me happy. Thank you, Mom and Dad; because of you, I have had the freedom to build upon your steadfast foundation, lifelong curiosity, and immense courage. To my brother, Christopher, thank you for your long-distance but unconditional support and your constant influx of levity. The three of you have always challenged me to be my best, whether in science, sport, music, or art; and I am grateful to have shared my adventures with you.
In closing, to all who have helped me arrive at this point, I will quote a certain friend. When I asked her how to write the conclusions to the thesis, she provided me with a succinct suggestion which I will amend:

To everyone: You are awesome, thank you, and goodnight.
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<tr>
<td>α-MSH</td>
<td>alpha-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>anterior-posterior</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
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<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BLA</td>
<td>basolateral nucleus of the amygdala</td>
</tr>
<tr>
<td>BnST</td>
<td>bed nucleus of the stria terminalis</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA(1-3)</td>
<td>cornu Ammonis</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>CRF-BP</td>
<td>corticotropin-releasing factor binding protein</td>
</tr>
<tr>
<td>c&lt;sub&gt;v&lt;/sub&gt;</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DRE</td>
<td>downstream response element</td>
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<tr>
<td>DREAM</td>
<td>downstream response element antagonist modulator</td>
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<tr>
<td>DV</td>
<td>dorsal-ventral</td>
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<td>EF-1</td>
<td>elongation factor-1</td>
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<td>EPM</td>
<td>elevated plus-maze</td>
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<td>Edinger-Westphal</td>
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<td>FGF-1</td>
<td>fibroblast growth factor-1</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
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<td>γ-aminobutyric acid</td>
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<tr>
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<tr>
<td>HPA</td>
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<tr>
<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>inferior colliculus</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>ID</td>
<td>injected dose</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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LC  locus coeruleus
MAP  microtubule-associated protein
MCH  melanin concentrating hormone
MeA  medial nucleus of the amygdala
MGN  medial geniculate nucleus
ML  medial-lateral
MN  mammillary nucleus
mPFC  medial prefrontal cortex
mPVN  magnocellular PVN
mRNA  messenger ribonucleic acid
MR  mineralocorticoid receptor
NGF  nerve growth factor
NMDA  N-methyl-D-aspartic acid
NPY  neuropeptide Y
NTS  nucleus of the solitary tract
OCT  optimal cutting temperature
OF  open field
OVLT  organum vasculosum of the lamina terminalis
PAG  periaqueductal grey
PFA  paraformaldehyde
POMC  proopiomelanocortin
pPVN  parvocellular PVN
PVN  paraventricular nucleus of the hypothalamus
PVT  paraventricular nucleus of the thalamus
SAL  saline
SCN  suprachiasmatic nucleus
SEM  standard error of the mean
SFO  subfornical organ
SOD  superoxide dismutase
SRE  serum response element
TBS  tris-buffered saline
TBS-T  tris-buffered saline with tween-20
TCAP  teneurin C-terminal associated peptide
TRH  thyrotropin-releasing hormone
Ucn  urocortin
VTA  ventral tegmental area
1 Chapter One: Introduction: The stress response and the search for novel neuropeptides

1.1 Abstract

The study of stress has spanned the greater part of the last century, and a particular milestone in the field was the publishing of Walter Cannon’s early work on homeostasis and Hans Selye’s work on the general adaptation syndrome in the 1930s. The work popularized the idea of the stress response, which involved autonomic, neuroendocrine, and behavioural responses to stimuli that could upset the homeostatic balance. Later work would identify the releasing factors involved in the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing factor (CRF), the hypothalamic activating peptide of the HPA axis, was sequenced in 1981, which started an explosion of stress-related research that identified the neuroanatomy of stress-related circuits, identified three more CRF-related peptides (the urocortins), and explored CRF’s role as a mediator of the stress response. Quite recently, a new distantly related but CRF-like peptide family was found, called the teneurin C-terminal associated peptides (TCAPs). The TCAP sequence possesses the characteristics of a bioactive peptide, and synthetic versions of the predicted TCAP-1 peptide possess both in vitro and in vivo effects, including reorganization of the cytoskeleton, neuroprotection under alkalotic conditions, and modulation of acoustic startle behaviour. However, much of TCAP-1’s in vivo characteristics were unknown, especially TCAP-1’s role in stress-like behaviours, an attribute suggested by TCAP-1’s sequence similarity to CRF and TCAP’s mRNA distribution in the brain. Therefore, the objective of this thesis is to determine if TCAP-1 can regulate CRF-mediated processes in the brain, including neuronal activation of brain areas, alteration of neuronal morphology, and/or modulation of stress-like behaviours.

1.2 Homeostasis, allostasis, and the stress response

In his seminal 1936 letter to Nature, Hans Selye (1936) described a non-specific syndrome that exists in rats after exposure to noxious stimuli such as cold, injury, or immune challenge, which he coined the “general adaptation syndrome”. This occurred in three parts: 1) the alarm reaction, in which there are a myriad of physiological and behavioural changes, such as: an increase in the immune response, disappearance of fat tissue, a decrease in body temperature, enlargement of
the adrenals, and sexual inhibition; 2) resistance, where small but repeated exposure to noxious stimuli will elicit physiological and behavioural changes that will allow the organs and tissues to return to a semblance of normality; and 3) exhaustion, in which chronic noxious stimuli cause the rats to be unable to cope with ongoing insults such that the rat succumbs to the effects of the stimuli, exhibiting symptoms similar to the alarm reaction (Selye, 1936).

Selye had developed his theories during his time at McGill University, and was heavily influenced by researchers such as Walter Cannon, who termed the “fight or flight response” and developed theories on homeostasis, and Claude Bernard, who coined the term “milieu interieur”. During the 1930s, the term “stress” had been used in passing in physiological circles (Viner, 1999), as well as by Walter Cannon, who used the term in 1935 to describe physiological responses as a result of homeostatic perturbation (Cannon, 1935). However, it was not until Selye that the “general adaptation syndrome” and “stress” became linked, and the theory was highly controversial throughout the 1940s and 1950s, such that even Cannon was a vocal opponent (Viner, 1999). Selye categorized stress into two extremes: “eustress” responses were beneficial or behaviourally activating responses to a stressor, whereas “distress” responses were damaging responses to a protracted stressor. Over the next few decades, Selye’s model would be altered and thoroughly transmuted by the time it became accepted as the norm. By the 1970s the word “stress”, by constant usage, became part of the normal lexicon (Viner, 1999), although by his own admission later in life, Selye admitted that the term “strain” would have been the better term, as in the field of physics, the applied force, or stress, on a material produces the strain. Modern stress theories now include the idea of “allostasis”, the changing of homeostatic setpoints in response to stimuli from the environment (McEwen and Wingfield, 2003).

Allostasis was first formulated by Sterling and Eyer (1988) as a reimagining of Cannon’s original theories on homeostasis (Day, 2005), and was later championed by Bruce McEwen (McEwen and Stellar, 1993). McEwen suggested that allostatic mechanisms are processes in the body that return the organism to a state of homeostasis and anticipate the demand for resources. This produces the “allostatic state”, in which the body maintains an altered level of allostatic mediators, such as heightened inflammatory cytokines or blood pressure (McEwen and Wingfield, 2003). Over time, strains on the systems create “allostatic load”, the wear-and-tear on a body from multiple noxious insults that could produce illness or other detrimental effects (“allostatic overload”) (McEwen and Stellar, 1993). McEwen hypothesized that allostatic
overload occurs in two forms: Type 1 occurs when energy demands exceed energy stores, and Type 2 occurs when energy intake is greater than energy demand. Type 1 allostatic overload elicits escape responses and other coping strategies, whereas Type 2 allostatic overload does not elicit an escape response and social conflict drives the detrimental effects.

However, the theory of allostatic overload is somewhat controversial (Dallman, 2003; Day, 2005) in that the allostatic overload theory suggests that any threat to homeostasis, however minor or beneficial, should be categorized as a stressor. As the whole brain is involved in maintaining homeostasis, then the whole brain should be seen as part of the stress circuit which is of no use to the stress neurocircuitry field (Day, 2005). Instead, Day suggests that stimuli that perturb homeostasis can elicit either selective responses (a small subset of tissues acting upon a minor challenge) or non-selective responses (a co-ordinated and simultaneous activation of tissues acting upon a real or perceived major challenge) to meet a challenge, and that Selye’s “non-specific” label on stress refers to responses that are common to all stressors, such as activation of the hypothalamic-pituitary-adrenal axis. Stress, therefore, can be redefined as an organism’s multi-system response to a challenge or stimuli that cannot be contained by the body’s homeostatic mechanisms (Day, 2005). Therefore, the stress response allows for appropriate reaction to novel and potentially harmful stimuli and allows the organism to interact with its environment.

Stressors are a part of everyday life. Organisms exist as a network of tissues and organs that must survive within a narrow range of physiological conditions. On a daily basis, organisms are subjected to a variety of noxious stimuli that could potentially be damaging to an organism, whether as a result of stressors such as predation, immune challenge, temperature, pH, exercise, lack of nutrition, or inter-species competition. In the human condition, stress has become a leading risk factor in a number of economically significant diseases, such as heart disease, mental illness, substance abuse, neurodegenerative disorders, and gastrointestinal disorders. However, stressors are not altogether detrimental; a small amount of stress is necessary for arousal and motivated behaviour.

The stress response is a multimodal system to protect the organism from perturbation. Harmful or stressful stimuli activate the sympathetic branch of the autonomic nervous system, inducing the secretion of catecholamines such as epinephrine (adrenaline) from the adrenal medulla and norepinephrine (noradrenaline) from the sympathetic ganglia neurons. Activation of the
sympathetic nervous system via brainstem efferents and the secretion of catecholamines are associated with the cardiovascular and visceral changes that prepare the body for the “fight or flight” response, which occurs rapidly after the onset of the stressor. Stressors will elicit the release of monoamines in the brain, such as norepinephrine, serotonin, and dopamine, which enhance the processing and integration of environmental or somatosensory stimuli. Perception of a stressor also results in activation of the hypothalamic-adrenal-pituitary (HPA) axis, the result of which is a slightly delayed secretion of glucocorticoids from the adrenal cortex. Tissues respond to glucocorticoids by increasing energy metabolism and availability so that the organism has sufficient energy to survive the stressful episode.

1.3 Stress and the HPA axis

The HPA axis is a seemingly simple feedback loop in its construction: the paraventricular nucleus of the hypothalamus (PVN) receives afferents from the sensory system and brainstem, secreting corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) during periods of stress, which projects to the median eminence and the hypophyseal portal system. CRF then reaches the anterior pituitary corticotropes, which are stimulated to secrete adrenocorticotropic hormone (ACTH) which enters the systemic circulation. ACTH then stimulates the adrenal cortex to secrete glucocorticoids (such as cortisol in humans and corticosterone in rodents), which will cause a host of different effects, including the increase of gluconeogenesis, mobilization of amino acids, increased lipolysis, and suppression of the immune system. Glucocorticoids will then feedback on the hypothalamus and pituitary to inhibit its own synthesis (Figure 1.1).

Basal glucocorticoid secretion follows a circadian rhythm, with concentrations peaking in the perceived morning. The rhythm is dependent on the suprachiasmatic nucleus (SCN), the brain’s internal clock (Herman et al., 2005), as lesions of the SCN abolish glucocorticoid rhythmicity (Moore and Eichler, 1972). Glucocorticoids exert a multitude of effects that could be potentially detrimental over long durations, and therefore its secretion is highly regulated. Glucocorticoids will feedback to acutely inhibit the release of CRF and chronically downregulate CRF and AVP expression in the PVN, and hence glucocorticoid secretion (Keller-Wood and Dallman, 1984). The “fast” feedback depends on the rate of glucocorticoid secretion, and is non-genomic in nature. This effect occurs within seconds but the feedback pathways are still not fully elucidated.
(Dallman, 2005), although this feedback is thought to involve inhibition of membrane-bound receptors that have rapid glutamatergic signalling to the PVN (Di et al., 2003). The more familiar “delayed” feedback system utilizes genomic changes that depend on the concentration of glucocorticoids (Herman et al., 2005) and works via inhibition of CRF and AVP synthesis and release in the PVN and ACTH and proopiomelanocortin (POMC) synthesis in the pituitary. Glucocorticoid negative feedback also occurs via limbic structures that have inputs to the HPA axis (Herman et al., 2003; 2005). Glucocorticoid actions occur by binding to two distinct cytosolic receptors. The first is the glucocorticoid receptor (GR), which is a type-II low-affinity receptor. GR is abundant in the brain and is only bound during periods of high glucocorticoid secretion, such as during stress. The mineralocorticoid receptor (MR), is a type-I high-affinity receptor with a much more limited distribution. The MR is bound even at basal levels of glucocorticoids, suggesting that the MR controls basal HPA axis tone (de Kloet et al., 1999).

Inhibition of the HPA axis also occurs without glucocorticoid input. The bed nucleus of the stria terminalis (BnST), medial preoptic nucleus, dorsomedial nucleus, and lateral hypothalamic area send γ-aminobutyric acid (GABA)-ergic projections to the PVN, which decrease CRF release, a process which is independent of glucocorticoids (Boudaba et al., 1996; Dong et al., 2001).

The HPA axis is not a static system; being essential for survival, its sensitivity changes in response to previous experience. Chronic stressors that are the same, and thus, predictable (for example, repeated exposure to restraint or white noise, known as a homotypic stressor), will decrease the responsiveness of the HPA axis to that stressor (Bhatnagar et al., 2002; Chowdhury et al., 2000; Girotti et al., 2006; Vallès et al., 2006). This attenuation, known as habituation, can be seen as a decrease in both glucocorticoid and ACTH levels (Culman et al., 1991; File, 1982; Helmreich et al., 1997) as well as a decrease in immunoreactivity of the immediate-early gene protein, c-Fos, in certain areas of the brain (Girotti et al., 2006). Habituation can be blocked by peripheral injection of MR antagonists (Cole et al., 2000), and GR expression in the hippocampus is correlated with the degree of habituation to a repeated stressor (Helmreich et al., 1997). The effects of habituation are reversible, although effects have been noted to last for at least 4-6 weeks, depending on the intensity of the stressor (Vallès et al., 2006).

The HPA axis also exhibits facilitation when exposed to a stressor and then presented with a different (also known as heterotypic) type of stressor. Heterotypic stressors will elicit increased
glucocorticoid and ACTH release, an effect that lasts for weeks (Akana et al., 1992; Helmreich et al., 1997; Kant et al., 1985; van Dijken et al., 1993). Both the GR and MR are involved in this potentiation, as GR and MR antagonists abolish this effect (Calvo and Volosin, 2001). Facilitation also increases GR and MR binding capacity in the hippocampus (van Dijken et al., 1993).

![Diagram of the hypothalamic-pituitary-adrenal axis](image)

**Figure 1.1: The hypothalamic-pituitary-adrenal axis.** Noxious stimuli will stimulate the parvocellular cells of the paraventricular nucleus of the hypothalamus (PVN) to release both corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), which enter the hypophyseal portal system. CRF and AVP stimulate the corticotropes of neuroendocrine tissues of the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH is then released into the systemic blood flow, where it stimulates the adrenal cortex to release glucocorticoids, such as cortisol (corticosterone in rodents). Glucocorticoids mobilize glucose in target tissues but also feedback on the brain to inhibit release of both CRF and ACTH, thus limiting its own release. Glucocorticoids will also feedback on limbic and other brain areas with input into the PVN to limit HPA axis output.
1.4 Neuroanatomy of the HPA axis

Activation of the HPA axis is accomplished through three pathways: local hypothalamic pathways, ascending input from the brainstem, and input from the circumventricular organs (Figure 1.2) (Ziegler and Herman, 2002). Meanwhile, the regulation of the HPA axis is highly influenced by a set of brain structures from the limbic system (such as the hippocampus, amygdala, and septum), thalamus, and cortex. These areas possess a multitude of interconnections with the HPA axis and each other and work synergistically to maintain HPA axis responsiveness to homeostatic challenges.

1.4.1 Excitatory input to the HPA axis

The hypothalamus consists of a network of distinct nuclei that are intimately interconnected, acting to secrete a variety of hormones into the hypophyseal portal system or through neurosecretory cells. For the stress response, the most important of these nuclei is the PVN, which is subdivided into distinct populations of neurons: the two largest are the magnocellular part (mPVN), which secretes oxytocin and vasopressin, and projects to the posterior pituitary; and the parvocellular part (pPVN), which secretes CRF, AVP, and thyrotropin-releasing hormone (TRH) into the hypophyseal portal system. Conventional wisdom would assume that the pPVN, as the main source of CRF in the HPA axis, would receive direct input from the stress-regulatory areas of the limbic system, but this is not the case (Herman et al., 2003; 2005). The pPVN is mostly confined to its own borders, with only a few connections directly from limbic structures. Instead, many stress-regulatory areas, such as the hippocampus, amygdala, and medial prefrontal cortex (mPFC), innervate the area surrounding the PVN, known as the peri-PVN, which sends inhibitory GABAergic projections to the pPVN (Herman et al., 2003).

The PVN also receives direct connections from relays such as the preoptic area, dorsomedial nucleus of the hypothalamus, BnST, and the nucleus of the solitary tract (NTS) (Boudaba et al., 1996; Cunningham and Sawchenko, 1988).

Circumventricular organ input to the PVN is achieved by the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT) (Figure 1.2). These two areas lack a blood-brain barrier and provide a mechanism for peripheral signals to reach the PVN. The SFO sends angiotensinergic input to the PVN and is sensitive to osmotic stressors (Larsen and
Mikkelsen, 1995; Plotsky et al., 1988), and activation of this pathway increases CRF and ACTH release (Plotsky et al., 1988). These areas are most likely more sensitive to systemic stressors.

Figure 1.2: Overview of excitatory inputs into the HPA axis. Excitatory inputs stimulate the parvocellular PVN to release CRF. Purple nuclei represent direct inputs to the PVN, green nuclei represent polysynaptic inputs to the PVN. Inputs from the nucleus of the solitary tract (NTS, A2) and the ventrolateral medulla (VLM, A1/C1) catecholaminergic efferents (NE) directly project to the PVN. The dorsal raphe nucleus (DRN) also sends sparse serotonergic (5-HT) connections directly to the PVN, but along with the locus coeruleus (LC), it also sends polysynaptic connections through other PVN-regulating areas. The amygdala (Amy) and lateral septum (LS) send excitatory input via disinhibition of GABAergic projections in the peri-PVN and bed nucleus of the stria terminals (BnST) area. Two circumventricular organs, the OVLT and the SFO directly innervate the PVN. Adapted from Ziegler and Herman et al., 2002.
The brainstem is a source of excitatory input in the HPA axis (Figure 1.2). Both the noradrenergic and serotonergic systems play roles in arousal and attention and it is therefore no surprise that they exert activating effects on the HPA axis. The brainstem sends both ascending noradrenergic input from the NTS and the locus coeruleus (LC) and serotonergic input from the dorsal raphe nucleus into the forebrain. The NTS is associated with the baroreflex response (Herman et al., 2003). It receives input from the mPFC, central nucleus (CeA) and medial nucleus (MeA) of the amygdala, although the CeA/MeA delivers opposing input (Dayas and Day, 2002). In addition, the NTS receives rich innervation from the area postrema, a circumventricular organ. The PVN receives direct input from the A2 noradrenergic cells around the NTS region (Cunningham and Sawchenko, 1988), and the NTS sends projections to the LC, CeA, and MeA (van Bockstaele et al., 1999). The NTS is sensitive to both psychological and physical stressors, although the area has been implicated as vitally important for the HPA axis response to physical or visceral challenges (Buller, 2003; Dayas et al., 2001; Herman and Cullinan, 1997; Pacak, 2000) and expresses GR and MR (Ahima and Harlan, 1990; Ahima et al., 1991). Another important source of excitatory input into the PVN is the LC, which is involved in arousal (Ziegler and Herman, 2002) and is one of the most stressor-sensitive areas in the brain (Herman and Cullinan, 1997). The LC receives input from the medial preoptic area, dorsal raphe, periaqueductal grey (PAG) (Luppi et al., 1995), and projects to forebrain regions such as the amygdala, mPFC, and hippocampus, hypothalamus, cerebellum, and cortex via the A6 noradrenergic cell group (Cunningham and Sawchenko, 1988; Grant and Redmond, 1981; Lehnert et al., 1998) which contains the highest concentration of noradrenergic cells in the brain (Foote et al., 1983). However, it does not send direct connections to the PVN (Cunningham and Sawchenko, 1988) and instead may activate stress-related pathways via activation of other areas connected to the PVN (Ziegler and Herman, 2002). The LC has a role in HPA axis modulation, as lesions reduce ACTH and corticosterone secretion in response to restraint (Ziegler et al., 1999). The dorsal raphe nucleus is a source of serotonergic input to the PVN. This nucleus receives input from the mPFC, hypothalamus, CeA, and medulla (Lee et al., 2003; Peyron et al., 1998) and sends projections to the lateral septum, striatum, PAG, hippocampus, thalamus, and BnST (Vertes, 1991). The dorsal raphe nucleus sends both direct and indirect projections to the PVN (Sawchenko and Swanson, 1983), and lesions of the raphe nuclei attenuate restraint-induced plasma ACTH (Jørgensen et al., 1998). The raphe nuclei contain dense bodies that
supply the brain with serotonin, which suggests that the raphe may indirectly influence the HPA axis via stress-regulating centres.

Unlike other components of the limbic system, such as the hippocampus or mPFC, the amygdala provides mostly excitatory input into the HPA axis through disinhibition of GABAergic relays to the PVN (Forray and Gysling, 2004). The amygdala is not a homogeneous structure, and distinct populations of nuclei are interconnected and respond to different stressors. Three nuclei will be discussed here: the MeA, CeA and BLA. The MeA receives innervation from the olfactory system, infralimbic cortex of the mPFC, and ventral subiculum of the hippocampus, and sends connections to the periaqueductal grey (PAG), ventral tegmental area (VTA), median raphe, medial preoptic area, and BnST (Canteras et al., 1995; Cullinan et al., 1993; Dong et al., 2001). The MeA expresses GR receptors, and to a lesser degree, expresses MR receptors (Ahima and Harlan, 1990; Ahima et al., 1991), and GR mRNA is upregulated in the MeA in times of chronic pain (Ulrich-Lai et al., 2006). The MeA has been implicated in the habituation process, as lesions block the attenuation to repeated restraint (Carter et al., 2004), and stimulation of the MeA increases plasma corticosterone levels (Dunn and Whitener, 1986). The CeA receives innervation from the PAG, somatosensory cortex, LC, and the BLA, (Dong et al., 2001; Prewitt and Herman, 1998) and sends projections to the BnST, lateral hypothalamic area, medial preoptic area, and NTS (Petrov et al., 1993; Prewitt and Herman, 1998). The CeA expresses both types of glucocorticoid receptors with high concentrations of GR (Ahima an Harlan, 1990), levels which are upregulated by chronic stressors (Ulrich-Lai et al., 2006). Lesions of the CeA cause a blunting of the ACTH response to immobilization stressors (Beaulieu et al., 1986). Interestingly, the MeA and CeA appear to be sensitive to different types of stimuli, as the MeA is sensitive to stimuli that are noxious but do not present a direct threat to the organism, such as restraint, forced swimming, noise, or open space (Dayas et al., 1999). These stimuli are known as psychological or emotional stressors. The CeA is sensitive to stimuli that are physical in nature, such as haemorrhage or immune challenge (Dayas et al., 2001). Finally, the BLA receives input from the thalamus and ventral subiculum (Canteras et al., 1995), but its efferent connections are not as extensive as the MeA or CeA. Instead, the BLA sends sparse projections to the anterior BnST and mPFC, but most of its connections are between amygdalar nuclei, including the MeA and CeA (Dong et al., 2001). Like the MeA, the BLA is activated by emotional or anticipatory stressors (Cullinan et al., 1995; Sawchenko et al., 2000) and is
particularly active in response to heterotypic stressors (Bhatnagar and Dallman, 1998). The BLA expresses both GR and MR (Ahima and Harlan, 1990; Ahima et al., 1991), and appears to have a role in the integration of stimuli.

The septum is thought to have a modulatory role on the HPA axis. The lateral septum is interconnected with the limbic system and hypothalamus, and sends projections to the peri-PVN area, medial preoptic area, and lateral hypothalamic area (Canteras and Swanson, 1992; Risold and Swanson, 1997). These areas are both GABAergic and glutamatergic (Risold and Swanson, 1996), indicating that the region may modulate HPA axis input by differential input into these areas. The region expresses both GR and MR (Calfa et al., 2006), and lesions of the lateral septum increased ACTH and corticosterone release after forced swimming (Singewald et al., 2011).

1.4.2 Inhibitory inputs into the HPA axis

The hippocampus is one of the most widely studied limbic structures and is associated with spatial reasoning, learning, and the consolidation of memory (Buwalda et al., 2005). The structure consists of Ammon’s Horn (CA1-CA3), dentate gyrus, fimbria, and the subiculum, and is divided into the dorsal (anterior) and ventral (posterior) parts. The hippocampus is innervated by the NTS, LC, amygdala, cortex, and thalamus, amongst other areas (Castle et al., 2005; Moga et al., 1995). Information generally enters through the entorhinal cortex and the dentate gyrus and is processed through the CA3 to CA1 before exiting through the subiculum. The hippocampus also receives input to the CA2 region from the supramammillary nucleus of the hypothalamus (Mercer et al., 2007). The hippocampus is mostly inhibitory on the HPA axis, sending excitatory connections to GABAergic neurons in the BnST and peri-PVN (Figure 1.3) (Cullinan et al., 1993). The hippocampus contains some of the highest concentrations of both GR and MR receptors in the brain (Ahima and Harlan, 1990; Ahima et al., 1991), suggesting that the hippocampus is a main feedback site for the HPA axis. For example, electrical stimulation of the hippocampus causes a decrease in glucocorticoid secretion (Dunn and Orr, 1984), lesions increase the sensitivity to mild stressors (Herman et al., 1995), and chronic stressors increase GR levels in the hippocampus (Mizoguchi et al., 2003).

The mPFC is often associated with the integration of cognitive and emotional information, and the consolidation of past and present stimuli. Like the hippocampus, the mPFC is a source of
inhibitory input to the HPA axis (Figure 1.3). It is involved in higher-order cognitive processes, such as goal-directed behaviour, decision making, and reward seeking (Walton et al., 2002). The mPFC is located in the anterior forebrain and although it has several subdivisions (anterior cingulate, prelimbic, and infralimbic cortices), they are poorly delineated. The mPFC is innervated by the BLA, receives both noradrenergic input from the LC and dopaminergic input from the ventral tegmental area (Bacon et al., 1996; Herman et al., 2003), and sends projections to the amygdala, BnST, paraventricular nucleus of the thalamus (PVT), and dorsal raphe (McDonald et al., 1996; Radley et al., 2009; Vertes, 2002). The mPFC expresses GR and MR and their expression is altered by chronic stressors (Diorio et al., 1993; Mizoguchi et al., 2003). Lesions of the mPFC cause elevations of both ACTH and corticosterone in response to a restraint stressor, but not ether (Diorio et al., 1993) suggesting that this area may discriminate between stressor modalities.

The thalamus is situated above the hypothalamus and is well positioned in the brain to exert effects on the stress response. Particularly important is the paraventricular nucleus of the thalamus (PVT), which is vital to the habituation response. The PVT receives connections from many stress-regulating nuclei, such as the ventral subiculum, infralimbic cortex, prelimbic cortex, BnST, NTS, LC, and raphe nuclei (Otake et al., 2002). In turn, the PVT sends projections to the BnST, SCN, MeA, CeA, BLA, and ventral subiculum (Moga et al., 1995). Lesions of the PVT block the habituation of the HPA axis to homotypic stressors without affecting the baseline stress response (Bhatnagar et al., 2002) and may block facilitation to heterotypic stressors (Bhatnagar and Dallman, 1998). The PVT expresses both glucocorticoid receptors (Ahima and Harlan, 1990; Ahima et al., 1991), and MR antagonists, but not GR antagonists, abolish habituation to repeated restraint (Cole et al., 2000).
Figure 1.3: Overview of inhibitory inputs into the HPA axis. Inhibitory inputs into the PVN prevent the release of CRF to the pituitary. Purple nuclei represent direct connections to the PVN, green nuclei represent polysynaptic input to the PVN. Inhibitory input from the hippocampus (Hi) exits through the ventral subiculum (vSub), which sends glutamatergic connections (+) to the GABA-rich (-) peri-PVN and bed nucleus of the stria terminalis (BnST) area, which inhibit the PVN. The medial prefrontal cortex (mPFC) also sends similar glutamatergic projections to the BnST and peri-PVN area. Adapted from Ziegler and Herman, 2002.

The HPA axis receives direct input from the BnST, which serves as a relay between the PVN and stress-regulating brain structures. The BnST is thoroughly subdivided and each brain area that innervates the BnST does so in a specific pattern, although there is considerable overlap (for an example, see Figure 1.4). The BnST has connections with the CeA, NTS, PAG, VTA, and the hypothalamus (Forray and Gysling, 2004). Notably, the BnST also receives noradrenergic input from the A1 and A2 noradrenergic cell groups in the brainstem (Forray and Gysling, 2004). The BnST sends mostly inhibitory inputs into the PVN (Boudaba et al., 1996; Cullinan et al., 1993), although electrical stimulation of the anterodorsal and fusiform subnuclei in the anterior BnST increase plasma corticosterone (Dunn, 1987).
Figure 1.4: Inputs into the BnST from the amygdala. Projections from the CeA or MeA to the BnST produce overlapping, but unique patterns. CeA projections are represented by red dots, and MeA projections are represented by blue dots. Adapted from Dong et al., 2001, BnST diagram adapted from Paxinos and Watson, 1998.

Together, excitatory inputs from the brainstem, circumventricular organs, septum, and amygdala, and inhibitory inputs from the hippocampus and mPFC (via the BnST and peri-PVN area), shape the HPA axis response to a variety of psychological and physical stressors. Physical stressors, such as ether inhalation, haemorrhage, or injection of inflammatory cytokines, activate catecholaminergic brainstem neurons which activate the PVN directly (Herman and Cullinan, 1997). On the other hand, psychological stressors, such as restraint and noise, are first processed by the limbic circuitry and the cortex before being relayed to the HPA axis, as interpretation of the stimulus requires integration of multiple sensory modalities before execution of the response. Activation of the HPA axis in this case requires inhibition of GABAergic connections to the PVN. Therefore, these areas serve to categorize and modulate the output of the HPA axis to form the appropriate physiological response.
1.5 Neuroanatomy of the behavioural stress response

Noxious stimuli have multi-pronged effects in that they activate neuroendocrine, autonomic, and behavioural responses. Perception of a stressor can result in anticipatory states modulated by the sympathomedullary system and HPA axis, such as the behavioural dimension known as anxiety. This state is the anticipatory behavioural response to a future stressor, whether real or imagined, and is exacerbated by continued presence of the stressor. It has also been linked to fear, the behavioural response to an identifiable potential threat that subsides shortly after its onset (Davis et al., 1997). Anxiety-like behaviour, which can include freezing, hypervigilance, risk assessment, avoidance of open spaces, xenophobia, and defensive behaviours, are measured by a variety of rodent tests (for a review, see Rotzinger et al., 2010) that produce stressful environments in which behaviours such as startle, movement, and approach-avoidance can be quantified. Tests of anxiety have been validated using anxiolytic and anxiogenic (anxiety-reducing and anxiety-inducing, respectively) drugs that have efficacy in humans, suggesting that the behaviour exhibited in the test is related to anxious states.

The amygdala is important in fear behaviours and emotional memory (Rodrigues et al., 2009), and it receives input from multiple sensory modalities and projects to limbic, hypothalamic, and brainstem nuclei. The amygdala is intimately connected to the central autonomic system components, such as the BnST, LC, NTS, parabrachial nucleus, and ventrolateral medulla, but also has connections to the limbic system and the HPA axis. The BLA serves as the input to the amygdaloid complex, whereas the CeA serves as a major output. Lesions of the BLA or CeA eliminate fear-potentiated acoustic startle (Davis et al., 1997; Oakes and Coover, 1997) but have no effect on unconditioned startle (Melia et al., 1992). Amygdala lesions also impair approach-avoidance behaviour in the elevated T-maze (Strauss et al., 2003), which can be interpreted as reduced anxiety-like behaviour; however another group found no effect in the elevated plus maze (EPM), a similar model (Treit et al., 1993). The same group also noted that amygdala lesion induced altered behaviours toward painful stimuli in the shock-probe burying test (Treit et al., 1993). As mentioned previously, the amygdala is also sensitive to glucocorticoids, as implants of corticosterone increased anxiety-like behaviour, an effect that was reversed by MR or GR antagonists (Myers and Greenwood-Van Meerveld, 2007). It has also been suggested that the amygdala is important for passive coping strategies (Roozendaal et al., 1997), and the amygdala
is well positioned to work in concert with the limbic system and brainstem efferents to modulate stress behaviours.

The septo-hippocampal pathway, consisting of the septum, fimbria-fornix, and the hippocampus, has also been implicated in regulating anxiety-like behaviour. The hippocampus is also bi-directionally linked to the BLA, and the septum is connected with the MeA and CeA, suggesting that the two areas may work in concert to regulate stress behaviours. Lesions in the ventral but not the dorsal hippocampus generally reduce anxiety-like behaviour in the EPM, behaviour that was not a result of deficits in spatial reasoning (Degroot and Treit, 2004; Kjelstrup et al., 2002). Tetrodotoxin lesions of the fimbria-fornix, an area generally important in learning, had anxiolytic effects in the EPM and Vogel conflict tests (Degroot and Treit, 2004). Lesions of the septum, a structure closely associated with the hippocampus, decrease anxiety-like behaviour in the EPM (Degroot and Treit, 2004; Treit et al., 1993) and shock-probe burying test (Degroot and Treit, 2004), although it potentiated unconditioned acoustic startle (Melia et al., 1992). MR antagonists have fast-acting effects in the hippocampus, reducing anxiety-like behaviour (Bitran et al., 1998; Smythe et al., 1997).

Another area that is closely linked with both the HPA axis and the limbic system is the mPFC, which is involved in decision making and cognitive functions. Lesions of the mPFC decrease anxiety-like behaviour in the EPM and social interaction tests (Gonzalez et al., 2000), although increased anxiety-like behaviour in the social interaction test was seen 5 weeks after lesion (Rangel et al., 2003). Inactivation of the mPFC produces similar anxiety-reducing effects in the Vogel conflict test (Resstel et al., 2008).

As reviewed previously, these areas integrate the response to psychological stress and express glucocorticoid receptors. Although glucocorticoids have been implicated in mediating stress-like behaviours such as freezing, memory consolidation, and fear (de Kloet, 2004), other peptide systems are involved in stress-like behaviours that are independent of HPA axis activation.

### 1.6 The role of corticotropin-releasing factor (CRF) family: mediators of stress

The existence of a factor that could be released by the median eminence to trigger pituitary hormone release was first hypothesized in the late 1930s (Harris, 1937). In the mid-1950s, two
groups identified that hypothalamic extracts contained a factor that could induce ACTH release from the pituitary (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). However, it was not until 1981 that Wylie Vale and colleagues determined the structure and sequence of ovine CRF (Spiess et al., 1981; Vale et al., 1981), and the sequence was subsequently determined in rats, pigs, man, and other species (for a review, see Rivier and Plotsky, 1986). The newly synthesized peptide could elicit ACTH release and behavioural activation in rats (Britton et al., 1982; Sutton et al., 1982; Vale et al., 1981). Stressor exposure increased CRF mRNA levels in the PVN (Imaki et al., 1991; Lightman and Young, 1988; 1989), and thus CRF was soon heralded as an endogenous mediator of stress-like responses. CRF co-localized with oxytocin, AVP, and neurotensin (Sawchenko et al., 1984) and CRF-immunoreactivity was found in the PVN but was also found centrally (i.e. outside of the hypothalamus) in areas such as the CeA, BnST, septum, NTS, LC, neocortex, and hippocampus (Figure 1.5) (Sakanaka et al., 1987; Swanson et al., 1983). Radioiodination of CRF with iodine-125 located high-affinity uptake sites in the anterior pituitary (De Souza et al., 1984; Wynn et al., 1984) as well as a pattern in the brain similar to CRF-immunoreactivity, suggesting that CRF had a neurotransmitter role (De Souza et al., 1985; Wynn et al., 1984). This was strengthened by experiments from the Nemeroff group showing that amygdala, striatum, and midbrain tissue could Ca\(^{2+}\)-dependently release CRF (Smith et al., 1986). Chronic stressors such as cold, or unpredictable stressors increased CRF-immunoreactivity in the LC and anterior hypothalamic area, and elicited CRF release from median eminence, arcuate nucleus, medial preoptic nucleus (Chappell et al., 1986), CeA (Merali et al., 1998), and hippocampus (Chen et al., 2004; 2006). These results suggest that whereas PVN CRF is vital for the HPA axis response (Beyer et al., 1988), initiation of stress-like behaviours may be regulated by central CRF actions that are independent of HPA axis activation (Britton et al., 1986a; 1986b).
Figure 1.5: Distribution of CRF receptors and CRF-like peptides. (Top) Distribution of the CRF₁ and CRF₂ receptors in the brain. (Bottom) Distribution of the CRF, Ucn 1, Ucn 2, Ucn 3, and TCAP in the brain. 7, facial nucleus; 10, vagus nucleus; 12, hypoglossal nucleus; AO, accessory olfactory nucleus; ARC, arcuate nucleus; BG, basal ganglia; BLA, basolateral nucleus of amygdala; BNST, bed nucleus of the stria terminalis; CB, cerebellum; CeA, central nucleus of amygdala; CgCx, cingulate cortex; EW, Edinger-Westphal nucleus; FrCx, frontal cortex; Hi, hippocampus; IC, inferior colliculus; LC, locus coeruleus; LDTg, lateral tegmental nucleus; LS, lateral septum; LSO, lateral superior olive; MeA, medial nucleus of amygdala; MnPO, medial preoptic nucleus; MS, medial septum; NTS, nucleus of the solitary tract; OB, olfactory bulb; OccCx, occipital cortex; OTu, olfactory tubercle; PAG, periaqueductal grey; ParCx, parietal cortex; PB, parabrachial nucleus; PF, perifrontal area; PG, pontine grey; PPTg, pedunculopontine tegmental nucleus; PVN, paraventricular nucleus of the hypothalamus; R, red nucleus; RN, raphe nucleus; SON, supraoptic nucleus; SC, superior colliculus; SN, substantia
nigra; Sp5n, spinal trigeminal nucleus; SPO, superior paraolivary nucleus; Thal, thalamus; VLM, ventrolateral medulla; VMH, ventromedial hypothalamic nucleus. Adapted from Reul and Holsboer, 2002.

1.6.1 CRF receptors

Two G protein-coupled receptors which activate adenylate cyclase were identified. The type 1 receptor (CRF₁) is a 415-amino acid residue protein that binds CRF with high affinity (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993) and belongs to the family B of G protein-coupled receptors, like those that bind calcitonin and growth hormone-releasing hormone (Chen et al., 1993). Eight splice variants have been identified (α, β, c-h) (Zmijewski and Slominski, 2010), although the effects of the CRF₁α isoform, which is involved in classical CRF signalling, will be reviewed here. A second distinct but structurally-related type 2 receptor (CRF₂) was later identified with 70% identity to the CRF₁ receptor sequence (Lovenberg et al., 1995b; Perrin et al., 1995), which is a 431-amino acid residue protein, but differs in the N-terminus from the CRF₁ receptor (Perrin et al., 1995). Although CRF is able to bind to the CRF₂ receptor, it preferentially binds the CRF₁ receptor. Two splice variants of the CRF₂ receptor were identified in rats and are differentially expressed as CRF₂(a) in the brain and CRF₂(b) in the periphery (Lovenberg et al., 1995a). A third isoform, the CRF₂(c) receptor, was later identified in humans, although not in any other species to date (Hauger et al., 2003; Kostich et al., 1998). The CRF₂(a) receptor will be discussed further. A binding protein, CRF-BP, was also identified in both the brain and periphery (Potter et al., 1991; 1992). The CRF-BP inhibits ACTH release and binds to CRF, modulating its effects (Behan et al., 1995).

The distribution of the CRF₁ receptor in the brain is widespread, for the most part overlapping with areas of CRF immunoreactivity (Figure 1.5). CRF₁ receptor mRNA is found throughout brain, including the anterior pituitary, layer IV of the cortex, parts of the olfactory pathway, hippocampus, BLA, MeA, medial septum, supramammillary nucleus, lateral hypothalamic area, parts of the brainstem, and cerebellum (Bittencourt and Sawchenko, 2000; Chalmers et al., 1995; van Pett et al., 2000). Stressors cause a biphasic change in pituitary CRF₁ receptor expression, decreasing mRNA 2 hours after and increasing mRNA 4 hours after stressor exposure (Aguilera et al., 2004). Under basal conditions, CRF₁ is not expressed in the PVN, although different stressors increase CRF₁ receptor mRNA in the PVN, such as lipopolysaccharide injection, immobilization (Rivest et al., 1995), haemorrhage, or footshock (van Pett et al., 2000).
Immobilization also causes a decrease in CRF<sub>1</sub> receptor mRNA in the cortex and BLA (Rivest et al., 1995). Repeated i.c.v. CRF administration increases CRF<sub>1</sub> receptor expression in the mPFC and hippocampus and downregulates CRF<sub>1</sub> receptors in the amygdala (Hauger et al., 2006). In contrast to the CRF<sub>1</sub> receptor, the CRF<sub>2</sub> has a more limited distribution. CRF<sub>2</sub> receptor mRNA is found in the lateral septum, ventromedial hypothalamic nucleus, MeA, dorsal and median raphe nuclei, parts of the BnST, hippocampus, olfactory bulb, NTS, arcuate nucleus, magnocellular PVN, and PAG (Bittencourt et al., 2000; Chalmers et al., 1995; van Pett et al., 2000).

Injections of CRF revealed that activation of the CRF receptors has a role in the stress response. As expected, intravenous (i.v.) CRF induced ACTH release by acting upon the anterior pituitary through activation of the CRF<sub>1</sub> receptor (Orth et al., 1983; Vale et al., 1981). Pharmacological studies showed that i.v. CRF<sub>1</sub> receptor antagonists that do not cross the blood-brain barrier, such as Astressin B, blocked the stressor-induced release of ACTH (Rivier et al., 2003). However, intracerebroventricular (i.c.v.) injection of CRF, a route of administration that reaches the brain parenchyma (Bittencourt and Sawchenko, 2000), also had central effects, as it elevated plasma catecholamines (Brown et al., 1982a; 1982b), increased mean arterial pressure and heart rate (Fisher et al., 1982), decreased growth hormone, luteinizing hormone (Ono et al., 1984), and gonadotropin-releasing hormone (Petraglia et al., 1987), and increased pituitary ACTH release (Brown et al., 1982b). Moreover, i.c.v. injection of CRF (for a review, see Dunn and Berridge, 1990) or intra-amygdalar injections of CRF (Daniels et al., 2004; Liang and Lee, 1988; Sajdyk et al., 1999) have well-documented effects increasing anxiety-like behaviours in rats. In addition, hypophysectomized rats, which lack the pituitary gland, are still behaviourally responsive to i.c.v. CRF (Berridge and Dunn, 1989; Eaves et al., 1985). Most of these effects were shown to be mediated by the CRF<sub>1</sub> receptor, as CRF<sub>1</sub> antagonists abolished behavioural responses to stressors (for a review, see Rotzinger et al., 2010), and CRF<sub>1</sub> receptor-deficient mice displayed reduced stressor-induced release of ACTH and corticosterone (Smith et al., 1998; Timpl et al., 1998), and decreased anxiety-like behaviour (Chotiwat et al., 2010; Contarino et al., 1999; Smith et al., 1998; Timpl et al., 1998). However, this effect could likely be a result of corticosterone deficiency in development because of impaired HPA axis signalling. However, a particularly elegant study supports that central CRF<sub>1</sub> is vital for the behavioural response: a mouse line with a conditional knockout of cortical and limbic CRF<sub>1</sub> receptor but intact pituitary CRF<sub>1</sub> receptor showed decreased anxiety-like behaviour but enhanced restraint-induced ACTH and
corticosterone release that persisted longer than controls (Müller et al., 2003). This indicates that pituitary CRF$_1$ receptor is vital for HPA axis activation, central CRF$_1$ receptor is important for HPA axis feedback, and reduced anxiety-like behaviour in conditional knockouts is HPA axis-independent. In addition, lentiviral knockdown of the CRF$_1$ receptor in the BLA (Sztainberg et al., 2010) or oligonucleotide injection into the CeA (Liebsch et al., 1995) reduced anxiety-like behaviour. These data support theories that the behavioural response to stress is mediated by extrahypothalamic CRF circuits and independent of pituitary CRF$_1$ receptors.

In contrast to the CRF$_1$ receptor, the role of central CRF$_2$ receptors is not as clear. I.c.v. injections of anti-Svg-30, a CRF$_2$ receptor antagonist, attenuated stress-like behaviour (Takahashi et al., 2001) and CRF-induced stress-like behaviour (Risbrough et al., 2003). Deletions of the CRF$_2$ receptor resulted in normal basal ACTH and corticosterone levels with enhanced ACTH and corticosterone responses to restraint (Bale et al., 2000; Coste et al., 2000; Preil et al., 2001) and prolonged elevated corticosterone after restraint (Coste et al., 2000). Deletion of the CRF$_2$ receptor in mice produced varying effects on stress-like behaviour, showing no effect (Coste et al., 2000) or an increase in stress-like behaviour (Bale et al., 2000; Chotiwat et al., 2010; Kishimoto et al., 2000). CRF$_1$ and CRF$_2$ receptor double knockout in mice abolished restraint- and CRF-induced ACTH and corticosterone (Bale et al., 2002; Preil et al., 2001), and the reduced HPA axis responsiveness attributed to CRF$_1$ deficiency could not be compensated for by CRF$_2$ receptor deletion.

It does not appear that the CRF$_1$-CRF$_2$ receptor relationship is purely antagonistic. HPA CRF$_1$ receptor activation may be responsible for ACTH and corticosterone output, whereas central CRF$_1$ receptor activation may be responsible for behavioural effects and the termination of HPA axis output. Meanwhile, the CRF$_2$ receptor may have a dual role depending on the phase of the stress response, as it increases the sensitivity to stressors in the short term and decreases the sensitivity to stress and inhibits the HPA axis in the long term after the stressor has abated (Gysling et al., 2004; Reul and Holsboer, 2002). In addition, CRF$_2$ receptor activation may induce stress-coping behaviours, such as grooming, exploration, and hypophagia (Coste et al., 2000).
1.6.2 The role of the urocortins in the stress response

Additional CRF-like peptides were also identified, which began with fish urotensin 1 and frog sauvagine (Lederis et al., 1982; Montecucchi et al., 1980). However, more closely related peptides soon emerged. First was urocortin 1 (Ucn 1), a 40 amino acid peptide with 45% identity to CRF which binds to the CRF₁ and CRF₂ receptors with high affinity (Vaughan et al., 1995) and binds to the CRF-BP (Behan et al., 1996). Ucn 1 is richly expressed in the non-preganglionic Edinger-Westphal (EW) nucleus, as well as the lateral olivary nucleus, and PAG (Figure 1.5) (Vaughan et al., 1995). In addition, Ucn 1-immunoreactivity was found in the PVN and supraoptic nucleus. However, Ucn 1-immunoreactive terminals were found in only a few nuclei bearing CRF₂ receptors, such as the lateral septum (Bittencourt et al., 1999), and i.c.v. injection of Ucn 1 induced activation of CRF₂-positive areas (Bittencourt and Sawchenko, 2000; Vaughan et al., 1995). However, Ucn 1 expression does not overlap with CRF₂ receptor expression, suggesting that other endogenous ligands must exist that bind to the CRF₂. Urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3), both of which are 38 amino acid residues long were then identified (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). Unlike Ucn 1, these peptides bind the CRF₂ receptor with high affinity, with little affinity for CRF₁ receptor. Ucn 2 is expressed in the PVN, arcuate nucleus, and LC, and i.c.v. injection induces activation in the BnST, PVN, CeA, and the NTS (Reyes et al., 2001). Ucn 3 is expressed in the BnST, MeA, medial preoptic area, and perifornical area (Figure 1.5) (Lewis et al., 2001). The urocortins added to the family of peptides that have the potential to influence central CRF receptors (Figure 1.6), and, by their expression in stress-regulating areas, the stress response.

The roles of the urocortins are also not fully elucidated. Ucn 1 injection induces the release of ACTH (Asaba et al., 1998; Vaughan et al., 1995) and corticosterone (Asaba et al., 1998), and increases anxiety-like behaviour (Jones et al., 1998; Moreau et al., 1997; Spiga et al., 2006). These actions are presumably through activation of the CRF₁ receptor, although these effects may be a result of exogenous Ucn 1 acting upon sites not normally associated with Ucn 1 activity. Although injection of Ucn 1 can activate the HPA axis, it is not likely an endogenous HPA axis activator; adrenalectomy, which normally elevates CRF output from the hypothalamus, had no effect on Ucn 1 levels, and Ucn 1-antiserum did not affect ACTH output (Oki and Sasano, 2004). Instead, endogenous Ucn 1 appears to be involved in modulating stress behaviour. Cells from the EW send Ucn 1-rich terminals to the lateral septum, and injections of
CRF<sub>2</sub> receptor antagonist into the area abolished immobilization or CRF-induced stress behaviours (Radulovic et al., 1999). Restraint, footshock, or acute pain increased Ucn 1 mRNA in the EW (Cespedes et al., 2010; Harris et al., 2006; Kozicz et al., 2001; Okere et al., 2010 Weninger et al., 2000), and EW Ucn 1-immunoreactivty remains elevated 18 h after stressor (Kozicz et al., 2001). Mice deficient for CRF but with intact CRF receptors showed normal anxiety-like behaviour to stressors, but this effect was blocked by CRF<sub>1</sub> receptor antagonists (Weninger et al., 1999), suggesting that endogenous Ucn 1 may be mediating this behaviour. Mice deficient in Ucn 1 showed normal stress-induced endocrine responses (Vetter et al., 2002; Wang et al., 2002) and normal (Wang et al., 2002) or increased anxiety-like behaviour (Vetter et al., 2002). In particular, Vetter et al. suggest that the increase in anxiety behaviour may be a result of decreased CRF<sub>2</sub> activation in the lateral septum.

![Diagram of CRF peptide family and its binding partners](image)

**Figure 1.6: The CRF peptide family and its binding partners.** CRF binds with high affinity to the CRF<sub>1</sub> receptor and CRF binding protein (CRF-BP), and with low affinity to the CRF<sub>2</sub> receptor. Ucn 1 binds to the CRF<sub>1</sub>, CRF<sub>2</sub>, and CRF-BP with high affinity. Ucn 2 and Ucn 3 are selective agonists for the CRF<sub>2</sub> receptor.
Injections of the CRF$_2$ receptor-specific agonists, Ucn 2 and Ucn 3, had no effect on the HPA axis (Pelleymounter et al., 2004), although other reports indicated that Ucn 2 (Maruyama et al., 2007) and Ucn 3 (Jamieson et al., 2006) injection induced ACTH release. Activation of the CRF$_2$ receptor has produced mixed results in behavioural tests. Injections of Ucns 2 or 3 increased stress-like behaviour (Pelleymounter et al., 2002, 2004; Risbrough et al., 2003; Skórzewska et al., 2011) or elicited delayed but reduced stress-like behaviour (Reul and Holsboer, 2002; Valdez et al., 2002, 2003; Venihaki et al., 2004; Zhao et al., 2007). However, aside from the NTS, i.c.v. Ucn 2 activated cell groups that do not strongly express CRF$_2$ receptors, such as the CeA, PVN, and BnST, and did not activate areas of CRF$_2$ receptor expression, such as the lateral septum or raphe nuclei (Reyes et al., 2001). Immobilization increased Ucn 2 expression in the PVN but not the LC (Tanaka et al., 2003), and restraint increased Ucn 3 mRNA in the MeA and perifornical area (Harris et al., 2006). Ucn 2 or Ucn 3 knockout in mice did not alter HPA axis output or anxiety-like behaviour, although Ucn 3 knockout blocked the acquisition of social memories (Deussing et al., 2010). A triple knockout of all three urocortins resulted in normal basal and post-stress anxiety-like behaviour, but increased anxiety-like behaviour when tested 24 hours later, indicating that Ucn knockout mice do not recover from stressor exposure, although there was no difference in post-stress corticosterone levels (Neufeld-Cohen et al., 2010). This supports the theory that the urocortins, via activation of the CRF$_2$ receptor, are important endogenous mediators of the behavioural stress response.

The urocortins represent peptides that preferentially bind to the CRF$_2$ receptor and have been associated with coping behaviour and the termination of stress behaviours during the recovery phase. Ucn 1, in particular, may have a role in stress adaptation through its CRF$_1$ and CRF$_2$ receptor activity, but it has been hypothesized that the Ucn 1 pathway from the EW may constitute a separate, but complementary system in concert with the CRF-PVN pathway (Kozicz, 2007). Endogenous Ucn 2 and Ucn 3 appear to have a role in recovery after stress, although it may not initiate the HPA axis or stress behaviours. Although, it should be noted that whereas injections of Ucn 2 have modulatory effects on behaviour, it does not activate CRF$_2$ receptor-rich areas, except the NTS, which suggests that the Ucn 2 (or Ucn 3) form predicted by cDNA may not be the endogenous form.
1.7 The HPA axis and behavioural stress responses: two sides of the same coin

Clearly, both the HPA axis response and the behavioural response allow the individual to cope and survive homeostatic threats. However, the circuitries responsible for HPA output and behaviour, although they overlap, are not necessarily the same. Adrenalectomy itself does not produce anxiolytic effects on the EPM (Bitran et al., 1998), although it blocks anxiety-like responses after a stressor (Calvo and Volosin, 2001). Loss of forebrain MR (including the mPFC, hippocampus, and amygdala) also does not induce anxiety-like behaviour (Berger et al., 2006), and overexpression of forebrain MR reduces anxiety-like behaviour (Rozenboom et al., 2007). Forebrain knockout of the GR indicates that HPA axis reactivity is still intact after chronic stressor (Furay et al., 2008), whereas GR is required for HPA axis negative feedback, and GR knockout mice exhibit less anxiety-like behaviour (Boyle et al., 2006). These results suggest that glucocorticoids are required for anxiety-like behaviours. However, the study by Müller et al. (2003) clearly demonstrated in CRF₁ receptor forebrain knockout mice that whereas HPA axis output was intact, anxiety-like behaviour was impaired, and that the central, but not pituitary CRF₁ receptors are required for anxiety-like behaviours. Together, these data suggest that although glucocorticoids produce anxiety-like states in the brain, they cannot do so without central CRF input, and therefore the central CRF system is vital for stress behaviours.

1.8 The search for novel CRF-like peptides: the teneurin C-terminal associated peptides

The CRF peptide family is well-conserved and phylogenetically old, consisting of four members with an ancient common ancestor. CRF and Ucnś 1-3 appear to have evolved after two genome duplications, one of which may have occurred before the formation of the vertebrate lineage (for a review, see Lovejoy and Jahan, 2006). Duplications have allowed for the evolution of new peptides to create families with similar, but not necessarily overlapping functions. Peptides are some of the oldest signalling molecules, being present in the simplest of organisms. Therefore, a gene duplication before the vertebrate divergence, providing CRF with a sister peptide lineage in both vertebrates and invertebrates, could be the basis for a phylogenetically old but previously undiscovered CRF-related family.
In an attempt to identify CRF-like paralogues, a Ucn 1 probe was used as a low-stringency screen on a rainbow trout cDNA library. Of the 600,000 clones that were screened, a fragment was identified belonging to the C-terminus of the teneurin-3 transmembrane protein (Qian et al., 2004). The screen identified a sequence encoding a 40-amino acid residue peptide, which was deemed “teneurin C-terminal associated peptide-3” (TCAP-3) for its position on the terminal end of the teneurin protein. Vertebrates possess four teneurin proteins (teneurins 1-4) and each have a TCAP sequence on the C-terminal end (TCAPs 1-4), consisting of peptides 40 or 41 amino acids long that are encoded by the terminal 31st exon of the teneurin gene (Figure 1.7). Sequence analysis revealed that the TCAP sequences have about 20% amino acid identity with CRF (Wang et al., 2005) and possess cleavage and amidation motifs befitting a bioactive peptide (Qian et al., 2004). The TCAPs are highly conserved, but are also phylogenetically old, suggesting that they are important in the development and survivability of a variety of species (Lovejoy et al., 2006).

**Figure 1.7: The CRF and TCAP families.** Alignment of the CRF family of peptides with the teneurin C-terminal associated peptides. Blue boxes represent amino acid identity to CRF family peptides. Green boxes represent amino acid similarity. Dashed line represents a possible relation from a common ancestor with the CRF peptide family. Adapted from Wang et al., 2005.
As their name suggests, TCAPs are closely associated with the teneurin proteins. Teneurins are large type II transmembrane proteins with their N-terminus on the cytosolic face and their C-terminus on the extracellular face. Teneurins were originally discovered in *Drosophila* as the *ten-mlodz* genes by two separate groups (Baumgartner et al., 1994; Levine et al., 1994), and were subsequently found in *C. elegans*, chicken, mouse, and humans. Teneurins appear to have a role in development, as mutations in *Drosophila* result in embryonic lethality (Baumgartner et al., 1994; Levine et al., 1994). Teneurins are expressed in the developing nervous system (Kenzelmann et al., 2008), visual system (Kenzelmann et al., 2008; Kenzelmann-Broz et al., 2010; Rubin et al., 2002) and developing limbs (Kenzelmann-Broz et al., 2010; Tucker et al., 2001), and appear to promote neurite growth, cell adhesion, and axon guidance (Young and Leamey, 2009).

TCAPs are active *in vitro*, producing effects in neuronal culture models. Synthetic TCAP-3 increased cAMP accumulation at low levels, modulated cell proliferation depending on concentration, decreased teneurin-1 gene expression at low levels and increased teneurin-1 gene expression at high levels in immortalized Gn11 neurons (Qian et al., 2004). Further study continued with synthetic TCAP-1. Like TCAP-3, TCAP-1 increased cAMP accumulation at low levels, but decreased cAMP accumulation at high levels, suggesting a modulatory effect (Wang et al., 2005). The increase of cAMP was not blocked by a Ucn 1 antagonist, suggesting that this effect was not occurring via CRF1 or CRF2 receptors. TCAP-1 could also dose-dependently inhibit forskolin-induced cAMP, and like TCAP-3, TCAP-1 modulated cell proliferation (Wang et al., 2005). TCAP-1 was neuroprotective in alkalotic environments *in vitro*, allowing TCAP-1-treated N38 immortalized neurons to survive at a pH of 8 or 8.4. TCAP-1 treatment prevented cell death via inhibition of necrotic, but not apoptotic cell pathways, increasing superoxide dismutase (SOD)-1 and increasing SOD copper chaperone and catalase (Trubiani et al., 2007). TCAP-1 could also affect neuron morphology. TCAP-1 treatment in N38 cells modulated the outgrowth of neurites, which are projections from the soma of the neuron in culture, such that long neurites got longer whereas short neurites got shorter (Al Chawaf et al., 2007a). In primary hippocampal culture, TCAP-1 administration induced an increase in neurite outgrowth and the fasciculation of neurites, showing that effects could occur in a non-immortalized culture (Al
Chawaf et al., 2007a). Modulation and reorganization of the neuron likely requires reorganization of the cytoskeleton, and TCAP-1 increased both β-tubulin and β-actin levels in N38 cells, and caused a reorganization of β-tubulin in neurons (Al Chawaf et al., 2007a). These data suggest that TCAP-1 is involved in axonal growth and morphology.

TCAP-1 also had intriguing in vivo effects. In situ hybridization studies in the rat revealed that TCAP-1 mRNA could be found throughout the brain, such as in the cortex, hippocampus, amygdala, ventromedial nucleus of the hypothalamus, subthalamic nucleus, olfactory bulb, brainstem, and cerebellum (Figure 1.5) (Wang et al., 2005). These areas are associated with the regulation and integration of the stress response, suggesting that TCAP-1 has a role in regulating the HPA axis or stress behaviours. To determine if TCAP-1 could affect behaviour, TCAP-1 was tested in the acoustic startle test, in which high startle responses are associated with increased anxiety (Yeomans and Frankland, 1995). Rats were separated out according to basal startle behaviour prior to injections, producing a low-startle group and a high-startle group. Injections of TCAP-1 into the BLA modulated acoustic startle behaviour, increasing startle in the low-startle group and decreasing startle in the high-startle group (Wang et al., 2005). Furthermore, i.c.v. injections for 5 days produced a decrease in startle behaviour 21 days after the last injection, showing that TCAP-1 has long-lasting behavioural effects (Wang et al., 2005). These behavioural effects, along with the distribution of mRNA in key limbic and regulatory brain areas, indicate that TCAP-1 has an important role in stress-related and anxiety-like behaviours, and it may do so via HPA axis modulation or interaction with the CRF system.

1.9 Objectives and Hypothesis

The TCAP family presents a newly elucidated but evolutionarily old lineage, as TCAPs have been found in every vertebrate genome that has been studied thus far. TCAP-1 has many documented effects in intracellular signalling, neuroprotection, and cytoskeleton regulation, but very little work had been done on its effects in vivo. I hypothesize that TCAP-1 is an intrinsic component of the stress response system and therefore, interacts with the CRF system.

By virtue of TCAP-1’s mRNA distribution in stress-regulating brain areas, its effects on acoustic startle, and its structural similarity to CRF, it was necessary to determine if TCAP-1 could regulate the effects of CRF, and ultimately the behavioural stress response in vivo. To do so, it was first important to determine TCAP-1’s effects on anxiety-like behaviours, given the ability
of extrahypothalamic CRF to mediate the behavioural response. Secondly, it was essential to
determine both uptake sites and neuronal activation sites in the brain, to determine if TCAP-1
mRNA expression and activity overlapped. Due to TCAP-1’s effects on the acoustic startle, it
was important to focus on limbic circuits, such as the hippocampus and amygdala, as well as
higher-order structures that process information, such as the mPFC. Finally, it was important to
determine a mechanism for such behavioural modifications, as TCAP-1’s acoustic startle effects
were still present long after TCAP-1 was eliminated from the system. TCAP-1’s role in
remodelling the neural cytoskeleton could promote long-term modifications in brain
connectivity. To realize these goals, the following objectives were accomplished:

1) Explored the effects of TCAP-1 on stress-induced anxiety-like behaviours in the rat

2) Determined the activation and uptake areas in the brain where TCAP-1 exerts its effects

3) Examined the mechanism underlying TCAP-1’s long-term changes in stress-sensitive
structures in the brain

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2 Chapter Two: TCAP-1 and its role in anxiety-like behaviours

Parts of this chapter have been published in modified forms in:


2.1 Abstract

Teneurin C-terminal associated peptide (TCAP)-1 mRNA has been localized in areas of the brain associated with the stress response, such as the hippocampus, amygdala, and hypothalamus, suggesting that TCAP-1 is ideally positioned to play a role in stress behaviours. Indeed, previous studies showed that injections of TCAP-1 into the basolateral nucleus of the amygdala modulated the acoustic startle response in rats, effects which were long-lasting. Previous in vitro experiments showing TCAP-1’s neuroprotective effects had indicated that TCAP-1 has its most pronounced effects in the presence of a stressor, and that TCAP-1 could have different acute and chronic effects. In the current studies, Wistar rats were injected with i.c.v. TCAP-1 either acutely (1 day) or repeatedly (5 days or 10 days) and then challenged with either corticotropin-releasing factor or restraint. Rats were then tested in the elevated plus maze and open field tests, which are standard rodent exploratory tests of anxiety. In the absence of a stressor, TCAP-1 increased exploratory behaviours and decreased risk assessment, enhancing the effect of mild stress, whereas in the presence of a stressor, TCAP-1 appeared to increase the anxiety state, enhancing the effect of severe stress.
2.2 Introduction

The novel neuropeptide family of the teneurin C-terminal associated peptides (TCAPs) have about 20% amino acid identity with corticotropin-releasing factor (CRF), the principal activating peptide of the hypothalamic-pituitary-adrenal stress axis (Qian et al., 2004). The TCAP family has four members (TCAPs 1-4) that are 40 or 41 amino acids long. Of these peptides, TCAP-1 mRNA is expressed in key forebrain and limbic structures in the brain, including the hippocampus, amygdala, hypothalamus, and cortex (Wang et al., 2005). These areas have been implicated in the regulation of stress behaviours, indicating that TCAP-1 expression is well-positioned to regulate or modify stress-related behaviour.

Synthetic TCAP-1 induces a number of bioactive effects, both in vitro and in vivo. In cell culture, TCAP-1 modulates cAMP accumulation and cell proliferation (Wang et al., 2005), influences neurite outgrowth in mouse immortalized hypothalamic culture (Al Chawaf et al., 2007a), and protects against alkalotic cell death by upregulation of superoxide dismutase (Trubiani et al., 2007). Behaviourally, TCAP-1 elicits significant and long-lasting effects in a test of anxiety known as the acoustic startle test. This is an unconditioned test that does not require learning or motivated behaviour. When injected into the basolateral nucleus of the amygdala, an area of the brain associated with fear, TCAP-1 modulated the response to the acoustic startle. TCAP-1-treated rats that initially had a low baseline startle had a relatively increased startle response; whereas TCAP-1-treated rats that initially had a high baseline startle had a relatively decreased startle response (Wang et al., 2005). Injections of TCAP-1 into the lateral ventricles produced long-lasting decreases in acoustic startle relative to saline-treated animals, an effect that lasted 21 days after the last TCAP-1 injection (Wang et al., 2005). Finally, TCAP-1 administration for 5 days can decrease CRF-induced anxiety-like behaviour in the acoustic startle response 21 days after the final TCAP-1 injection (Tan et al., 2008). Together, these results indicated that TCAP-1 may not have effects under basal conditions, and that conditions that induce stress, such as alkaline conditions in culture or noxious auditory stimuli, may be required to see TCAP-1’s stress-modulating effects.

The elevated plus maze (EPM) and the open field test (OF) are two widely-used rodent tests of anxiety. These tests have been used to investigate the role of peptides (for example, CRF, cholecystokinin, urocortin, substance P, arginine vasopressin, and oxytocin) in anxiety behaviour.
(for a review, see Rotzinger et al., 2010). In particular, CRF produces anxiogenic effects in tests such as the acoustic startle, social interaction, and EPM tests (for a review, see Dunn and Berridge, 1990). Repeated restraint in a Plexiglas tube has also been used to induce anxiety-like behaviour in social interaction (Albonetti and Farabollini, 1993; Doremus-Fitzwater et al., 2009), although this treatment induces habituation of the stress response in the EPM (Jaferi and Bhatnagar, 2007; Thorsell et al., 1999) and light-dark box (Cancela et al., 1995), producing no effect or anxiolytic effects.

A “stressed” state may be required to potentiate the effects of TCAP-1 on behaviour, and therefore, injections of CRF or repeated restraint in a Plexiglas tube were used. We have subsequently shown that TCAP-1 may have a role in CRF-induced stress behaviours, as pretreatment with intravenous (i.v.) TCAP-1 for 5 days resulted in modulation of CRF-induced behaviour, depending on the route of CRF administration. In the OF, i.v.-administered TCAP-1 attenuated the effects of i.c.v.-administered CRF, whereas it potentiated the effects of i.v.-administered CRF (Al Chawaf et al., 2007b).

The results of the acoustic startle test suggested that TCAP-1 has a role in stress-related behaviours. Few behavioural experiments had been attempted previously; therefore, I investigated the role of both acute and repeated injections of TCAP-1 on stress (via CRF or restraint)-induced behaviours in the EPM and OF. The effects of TCAP-1 on these behavioural experiments are critical to formulate the questions postulated in the rest of the thesis.

2.3 Materials and Methods

2.3.1 Animals

Male Wistar rats weighing 250-275 g were obtained from Charles River Laboratories (Montreal, QC). Rats were singly housed in Plexiglas shoebox cages under standard laboratory conditions (12:12 h light:dark cycle, lights on at 0700 h, temperature 21 ± 1 °C) with food and water available ad libitum. Rats were given one week to acclimatize to laboratory conditions before surgery. All procedures were approved by the University of Toronto Animal Care Committee in accordance with the Canadian Council on Animal Care.
2.3.2 Surgery

Male Wistar rats were anesthetized with isoflurane (3% induction, 2-3% maintenance in 100% O₂) and fit into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Analgesics (0.5 mg/kg buprenorphine) were administered subcutaneously at the beginning of surgery. A midline incision was made along the top of the head, exposing bregma. A 22-gauge guide cannula (Plastics One, Roanoke, VA, USA) was implanted into the right lateral ventricle using the following flat-skull coordinates: AP −1.0 and ML −1.4 from bregma, DV −2.7 from dura (Paxinos and Watson, 1998). The guide cannula was secured to the skull using four jewellers’ screws and dental cement. The opening of the cannula was covered by a removable cannula dummy. The wound was treated with Hibitane (Pfizer Animal Health, Kirkland, QC) to prevent infection. Animals were kept under warm lamps until regaining consciousness and provided wet chow with Nutri-Cal (Vetoquinol USA, Fort Worth, TX) in a clean cage for 12 h. Rats were then transferred to a clean cage and were given one week to recover from surgery.

2.3.3 Peptides

Synthetic mouse TCAP-1 was synthesized at 95% purity using f-moc-based solid phase synthesis (American Peptide Company, Sunnyvale, CA). For the acute TCAP-1 administration, TCAP-1 stocks were prepared to a concentration of 2.1 × 10⁻⁴ M (equivalent to 1 μg/μl TCAP-1) by treating lyophilized TCAP-1 with ammonium hydroxide vapour, then dissolving in sterile saline. For the acute TCAP-1 treatment, the dose was based on the molar equivalent of 3 μg of CRF (630 pmol/rat). For the repeated TCAP-1 treatments, TCAP-1 stocks were prepared to a concentration of 10⁻⁴ M, and the dose was 300 pmol/rat. CRF (Sigma–Aldrich, Oakville, ON) was dissolved in sterile saline at a concentration of 1 μg/μl.

2.3.4 Injection procedure

Injections were given between 0800 and 1200 h. Lateral ventricle (i.c.v) injections were delivered at a rate of 2 μl/min by a Hamilton syringe with a pump (Razel Scientific Instrument Inc., Stamford, CT) connected via PE-50 tubing to a 28-gauge stainless steel injector that extended 1 mm below the cannula guide tip. After injection, the injector was left in place for 60 s following infusion to prevent backflow up the cannula.
2.3.5 Cannula placement verification

Cannula placement was confirmed upon sacrifice of the animal. The acute administration and 5-day treatment rats were sacrificed under CO₂ gas. Rats were then decapitated and their brains were i.c.v. injected with 1 µl of 1% methylene blue before their brains were retrieved. The brains were then obtained and fixed in 4% formaldehyde. 24 h later, brains were sliced coronally with a razor blade to expose the lateral ventricles. Rats were considered to have a proper cannula placement if methylene blue was found in the lateral ventricles. Rats with improper cannula placement were removed from the sample.

The 10-day treatment rats were deeply anesthetized under 3% isoflurane and quickly decapitated. The brain was obtained and placed in Golgi impregnation solution (see Chapter 4). Brain sections from the Golgi stain procedure were viewed under a microscope and cannula placement was verified by identifying a transection of the cortex and corpus callosum by the cannula tract into the right lateral ventricle. Rats with improper cannula placement were removed from the sample.

2.3.6 Elevated Plus Maze (EPM)

The elevated plus maze test (EPM) was conducted using a standard plus maze apparatus elevated 65 cm above the floor (Figure 2.1A). It consisted of two arms (50 cm x 10 cm) enclosed by black Plexiglas walls 40 cm high (i.e. the “closed arms”), and two arms (50 cm x 10 cm) which were open and not enclosed (i.e. the “open arms”). Each pair of arms was separated by 180° and connected by a centre platform (10 cm x 10 cm). The entire apparatus was painted with matte black paint and the maze was illuminated by two dim red lights (25 W bulbs) suspended above the two open arms. The floor around the maze was covered in dull black material to avoid shine that would cause background noise for the tracking software.
Figure 2.1: Schematics of the elevated plus maze (EPM, Fig. 2.1A) and open field (OF, Fig. 2.1B) tests. In the EPM, the maze is arranged in a cross with two closed arms with unscalable walls (thick lines) and two open arms (thin lines) that are elevated 65 cm from the ground. Rats are individually introduced to the middle of the maze facing an open arm and are allowed to freely explore for 5 min. Rats will generally avoid the open arms of the maze, and anxiolytic treatments will increase both open arm time and entries. In the OF, rats are allowed to freely explore in a novel box for 10 or 60 min. Rats will generally avoid the centre of the maze and remain in the perimeter. Anxiolytic treatments will increase the amount of time and entries in the centre.

For each test, rats were placed on the centre platform facing an open arm. Rats were allowed to freely explore the maze for 5 min and movements were recorded from above using a digital camera suspended above the centre of the maze. Rat movements were tracked, quantified, and compiled using an Ethovision video tracking system (Noldus Information Technology). Only data from rats that completed the full 5 min without falling off the maze were retained for the results. Supplementary behaviours, such as rearing (the rat assumes a vertical posture standing on its back two legs), head-dipping (the rat places four feet in the open arm and directs its head below the open arm looking at the floor below), or stretched-attend (the rat’s two hind legs remain stationary and the rat makes a forward locomotory motion with its front legs, stretching its body, and retreats back to a more compact position with its hind legs never changing position) were manually counted by an observer blind to the study treatments. The maze was cleaned with mild soap and ethanol between tests.
2.3.7 Open Field Test (OF)

The open field test (OF) apparatus consisted of a 50 cm x 50 cm arena with 40 cm high walls made of black particle board (Figure 2.1B). A 30 cm x 30 cm square in the centre of the open field was defined as the centre zone for data analysis. The apparatus was illuminated by either dim red lights [25 W bulbs] or white lights [100 W bulbs]). Rat movements were tracked, quantified, and compiled using Ethovision.

For each test, rats were placed in the centre of the arena. Rats were allowed to freely explore the maze for 10 min or 60 min. Supplementary behaviours (rearing) were manually counted by an observer blind to the study treatments. The maze was cleaned with mild soap and ethanol between tests.

2.3.8 Ethovision parameters

To maintain the same parameters for the EPM test, an initial “background” picture was taken of the maze before each round of tests to calibrate the maze. A small white cardboard cross (four 2.5 cm arms extending from a 10 cm x 10 cm centre) was placed in the middle of the maze to ensure that when the rat had extended all four paws across the threshold of either the open or closed arm, the Ethovision program would construe this as a true arm entry. The “arena” was determined by outlining the “background” picture with extra room allowed for the open arms, so that the rat signal would not be lost if the rat head-dipped over the open arm. To calibrate the distance moved, a line was drawn from tip to tip of the white cardboard cross (i.e. 15 cm). For the OF, a 30 cm x 30 cm square was introduced to the initial “background” before each round of tests to calibrate the maze and ensure the same “centre” area could be acquired regardless of changes to the camera position. To calibrate the distance moved in the OF arena, a line was drawn diagonally from one corner of the maze to the other (i.e. 70.7 cm). Both cardboard backgrounds were removed prior to testing in the maze, so that the entire arena had a uniform black surface.

During quantification of both the EPM and the OF, rat detection (white on black) was set to gray scaling, and the image was filtered, first with erosion (2 pixels) and then dilation (2 pixels) to eliminate background noise. 2 cm of minimum movement was required to be scored as genuine locomotion.
2.3.9 Experiment 1: Acute effects of TCAP-1 treatment

A timeline schematic of Experiment 1 can be found in Figure 2.2. Male Wistar rats (n = 158) were cannulated into the right lateral ventricle. After surgery, rats were handled for 5 min per day for five consecutive days and introduced to mock injections. On test day, rats were first given an i.c.v. injection of either saline or 630 pmol (in 3 µl) of TCAP-1. After waiting 60 s to prevent backflow up the cannula, rats were given a second i.c.v. injection of saline (0 µg CRF), 1 µg CRF, or 3 µg CRF. Rats were returned to their home cages in the colony for 2 h. Rats were then tested on the EPM, and returned to the colony upon completion of the test.

Figure 2.2: Schematic of Acute TCAP-1 experiment. On Day 1, rats were given an i.c.v. injection of 630 pmol (3 µg) of TCAP-1, followed directly by an i.c.v. challenge of 0, 1 or 3 µg CRF and were returned to the colony. Two hours later, rats were tested on the EPM. On Day 4, the same rats were again treated with i.c.v. TCAP-1 (630 pmol, 3 µg), followed directly by a challenge of 0, 1, or 3 µg CRF (i.c.v.) and returned to the colony. Two hours later, rats were tested on the OF.
Three days after the EPM test, rats were again injected with either saline or 630 pmol of TCAP-1, followed by either saline, 1 µg CRF or 3 µg CRF. Rats were returned to the colony for 2 h. Rats were then tested in the OF and returned to the colony upon completion of the test. There were 6 treatment groups; 3 of these were controls: saline injection with saline injection (“SAL+CRF (0 µg)”), saline injection with 1 µg CRF injection (“SAL+CRF (1 µg)”), and saline injection with 3 µg CRF injection (“SAL+CRF (3 µg)”; 3 of these were experimental groups: TCAP-1 injection with saline injection (“TCAP+CRF (0 µg)”), TCAP-1 injection with 1 µg CRF (“TCAP+CRF (1 µg)”), and TCAP-1 injection with 3 µg (“TCAP+CRF (3 µg)”).

2.3.10 Experiment 2: Effects of 5-day repeated TCAP-1 pre-treatment

A timeline schematic of Experiment 2 can be found in Figure 2.3. Male Wistar rats (n = 79) were cannulated into the right lateral ventricle. After surgery, rats were allowed to recover for 1 week. Rats were then i.c.v.-injected for 5 consecutive days (Days 1-5) with either saline or 300 pmol (in 3 µl) of TCAP-1. Rats were then allowed to rest for 1 week before behavioural tests. On Day 12, rats were injected with an acute challenge of saline (0 µg of CRF), 1 µg, or 3 µg of CRF (i.c.v.). Rats were then returned to the colony for 30 min. The rats were then individually tested in the EPM and returned to the colony.

On Day 15, three days after the EPM, rats were again administered i.c.v. saline (0 µg of CRF), 1 µg, or 3 µg of CRF. Rats were returned to the colony for 30 min. The rats were then tested in the OF and returned to the colony. There were 6 treatment groups; 3 of these were controls: saline pre-treatment with acute saline injection (“SAL+CRF (0 µg)”), saline pre-treatment with acute 1 µg CRF injection (“SAL+CRF (1 µg)”), and saline pre-treatment with acute 3 µg CRF injection (“SAL+CRF (3 µg)”; 3 of these were experimental groups: TCAP-1 pre-treatment with acute saline injection (“TCAP+CRF (0 µg)”), TCAP-1 pre-treatment with acute 1 µg CRF injection (“TCAP+CRF (1 µg)”), and TCAP-1 pre-treatment with acute 3 µg CRF injection (“TCAP+CRF (3 µg)”).
Figure 2.3: Schematic of 5-Day Pre-Treatment Administration experiment. Rats were treated daily with i.c.v. TCAP-1 (300 pmol) on Days 1-5. On Day 12, rats were injected (i.c.v.) with a challenge of 0, 1, or 3 µg CRF. Rats were returned to the colony for 30 min and then tested on the EPM. On Day 15, rats were injected (i.c.v.) with a challenge of 0, 1, or 3 µg CRF. Rats were returned to the colony for 30 min and then tested in the OF.

2.3.11 Experiment 3: Effects of 10-day repeated TCAP-1 treatment

A timeline schematic of Experiment 3 can be found in Figure 2.4. Male Wistar rats (n = 30) were cannulated into the right lateral ventricle. After surgery, rats were allowed to recover for 1 week. Rats were then injected (i.c.v.) with either saline or 300 pmol TCAP-1 for 10 consecutive days. In the afternoon of each of these 10 consecutive treatment days (1300-1500 h) rats were either handled lightly and allowed to run through the restraint tube (for rats to experience the novelty of the tube) as a non-stressed control, or were subjected to 2 h per day of restraint stress in a clear Plexiglas tube (length = 15.8 cm, interior diameter = 7 cm) in their home cages.

24 h after the last injection/restraint day, rats were tested on the EPM. Rats were then sacrificed and the brains were subjected to a Golgi impregnation study (see Chapter 4). There were 4 treatment groups: saline treatment with no restraint (“SAL”), TCAP-1 treatment with no restraint (“TCAP”), saline treatment with restraint (“SAL+Restraint”), and TCAP-1 treatment with restraint (“TCAP+Restraint”).
Figure 2.4: Schematic of 10-Day Repeated Administration experiment. Rats were treated each morning with i.c.v. TCAP-1 (300 pmol) and subjected to restraint stress in a Plexiglas tube for 2 h in the afternoon for 10 consecutive days. Rats were tested on the EPM on Day 11.

2.3.12 Data analysis

Behaviour in the EPM and OF was quantified using Ethovision software. Raw data was transferred to Microsoft Excel for sorting. Statistical analysis of the data was carried out with GraphPad Prism 4.0. For the EPM, “percent open arm time” was calculated as \(\frac{\text{open arm time}}{\text{open arm time} + \text{closed arm time}} \times 100\), and “percent open arm entries” was calculated as \(\frac{\text{open arm entries}}{\text{open arm entries} + \text{closed arm entries}} \times 100\). Data was converted to a percent of control of the corresponding saline group at each dose of CRF or restraint. Values were analyzed by a one-sample t-test against a hypothetical mean of 100%.

2.4 Results

2.4.1 Experiment 1: Acute effects of TCAP-1 treatment

As expected, i.c.v. injections of either 1 µg or 3 µg CRF produced significant dose-dependent increases in anxiety-like behaviour in the EPM and OF tests (Table 2.1) as measured by one-way ANOVA. CRF significantly decreased percent open arm entries (\(F_{2,63} = 3.507, p = 0.036\)), percent open arm time (\(F_{2,63} = 4.474, p = 0.0152\)), rearing (\(F_{2,63} = 7.43, p = 0.0013\)), and head-dipping (\(F_{2,63} = 5.239, p = 0.0079\)) in the EPM. CRF also significantly decreased centre entries (\(F_{2,75} = 8.28, p = 0.0006\)), centre time (\(F_{2,75} = 8.928, p = 0.0003\)), centre distance travelled (\(F_{2,75} = 9.689, p = 0.0002\)), total distance travelled (\(F_{2,75} = 5.595, p = 0.0054\)), and rearing (\(F_{2,75} =
15.23, p < 0.0001) in the OF. These data indicate that CRF could serve as suitable positive controls for TCAP-1-treated groups.

Table 2.1: Experiment 1: Acute injections of CRF are anxiogenic on the EPM and OF

<table>
<thead>
<tr>
<th></th>
<th>SAL+CRF (0 µg)</th>
<th>SAL+CRF (1 µg)</th>
<th>SAL+CRF (3 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Open Arm Entries (%) *</td>
<td>33.8</td>
<td>24.5</td>
<td>18.2</td>
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<td>% Open Arm Time (%) *</td>
<td>37.4</td>
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</tr>
<tr>
<td>Rearing **</td>
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<td>8.7</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Stretched-Attend</td>
<td>3.9</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>OF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centre Entries ***</td>
<td>37.5</td>
<td>30.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Centre Time (s) ***</td>
<td>113.8</td>
<td>92.4</td>
<td>41.6</td>
</tr>
<tr>
<td>Centre Distance (cm) ***</td>
<td>769.7</td>
<td>576.2</td>
<td>292.8</td>
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<tr>
<td>Total Distance (cm) **</td>
<td>2782.8</td>
<td>2532.0</td>
<td>1914.7</td>
</tr>
<tr>
<td>Rearing ***</td>
<td>48.4</td>
<td>37.0</td>
<td>14.7</td>
</tr>
</tbody>
</table>

(*p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA).

Figure 2.5: Acute TCAP-1 on CRF-induced behaviour in the EPM. Percent of control data for (A) percent open arm entries and (B) percent open arm time. TCAP-1-treated groups are presented as a percentage of saline-treated controls injected with a challenge of 0, 1, or 3 µg CRF. Acute TCAP-1 had no effect on open arm measures in the EPM at any CRF dose. Bars represent mean percent of control + SEM.
Figure 2.6: Acute TCAP-1 on CRF-induced behaviour in the OF. Percent of control for (A) centre entries, (B) centre time, (C) centre distance travelled, and (D) total distance travelled. Acute TCAP-1 had no effect on centre measures, although TCAP-1 in the absence of a stressor (TCAP+CRF (0 µg)) increased total distance travelled in the OF. Bars represent mean percent of control + SEM (***p < 0.001).

In the EPM, data from the SAL+CRF (0 µg), SAL+CRF (1 µg), SAL+CRF (3 µg) control groups (n = 29, 18, 19, respectively) and from the TCAP+CRF (0 µg), TCAP+CRF (1 µg), TCAP+CRF (3 µg) experimental groups (n = 31, 22, 21, respectively) were obtained. TCAP-1 group data was converted to a percent of control compared to the mean of the corresponding SAL+CRF group at each dose of CRF (0, 1, or 3 µg). Several measures were investigated: percent open arm entries, percent arm time, stretched-attend postures, head-dipping, and rearing. In percent open arm entries (Figure 2.5A), TCAP-1 at any concentration of CRF (0, 1, or 3 µg) had no effect relative to controls treated with CRF (0, 1, or 3 µg) (t = 1.365, p = 0.182; t = 1.212,
p = 0.239; t = 0.0866, p = 0.932, respectively). Similarly, in percent open arm time (Figure 2.5B), TCAP-1 with CRF treatment (0, 1, or 3 µg) was not significantly different from its respective control (t = 1.136, p = 0.265; t = 0.082, p = 0.935; t = 0.246, p = 0.808, respectively). In exploratory behaviours, such as rearing, TCAP-1 had no effect relative to controls at 1 or 3 µg treatments of CRF (Table 2.2; t = 0.741, p = 0.467 and t = 0.958, p = 0.35), however, TCAP-1 had a significant effect of increasing rearing behaviours by 11.8% relative to control in the absence of a stressor (i.e. TCAP+CRF (0 µg)) (t = 2.436, p = 0.021). TCAP-1 had no effect on stretched-attend postures (Table 2.2; TCAP+CRF (0 µg): t = 1.922, p = 0.0642; TCAP+CRF (1 µg): t = 1.239, p = 0.229; TCAP+CRF (3 µg): t = 0.398, p = 0.695) or head-dipping behaviours (Table 2.2; TCAP+CRF (0 µg): t = 0.226, p = 0.823; TCAP+CRF (1 µg): t = 0.820, p = 0.422; TCAP+CRF (3 µg): t = 0.530, p = 0.602).

<table>
<thead>
<tr>
<th></th>
<th>TCAP+CRF (0 µg)</th>
<th>TCAP+CRF (1 µg)</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>EPM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rearing</td>
<td>111.8%*</td>
<td>108.40%</td>
<td>108.80%</td>
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<td>Head-Dipping</td>
<td>97.60%</td>
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</tr>
<tr>
<td>Stretched-Attend</td>
<td>129.50%</td>
<td>121.60%</td>
<td>106.40%</td>
</tr>
<tr>
<td><strong>OF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rearing</td>
<td>113.90%</td>
<td>102.30%</td>
<td>103.50%</td>
</tr>
</tbody>
</table>

(*p < 0.05)
In the OF, data from the SAL+CRF (0 µg), SAL+CRF (1 µg), SAL+CRF (3 µg) control groups (n = 36, 22, 20, respectively) and from the TCAP+CRF (0 µg), TCAP+CRF (1 µg), TCAP+CRF (3 µg) experimental groups (n = 34, 23, 23, respectively) were obtained. TCAP-1 group data was converted to a percent of control compared to the mean of the corresponding SAL+CRF group at each dose of CRF (0, 1, or 3 µg). Several measures were investigated: centre entries, centre time, centre distance, total distance, and rearing. TCAP-1 did not affect centre entries at any concentration of CRF (0, 1, or 3 µg) relative to controls (Figure 2.6A; t = 1.246, p = 0.222; t = 0.354, p = 0.727; t = 1.474, p = 0.155, respectively). TCAP-1 also did not affect centre time (Figure 2.6B; TCAP+CRF (0 µg): t = 0.806, p = 0.426; TCAP+CRF (1 µg): t = 0.0743, p = 0.941; TCAP+CRF (3 µg): t = 1.635, p = 0.116) or centre distance (Figure 2.6C; TCAP+CRF (0 µg): t = 1.091, p = 0.283; TCAP+CRF (1 µg): t = 1.048, p = 0.306; TCAP+CRF (3 µg): t = 1.738, p = 0.0962). However, TCAP-1 produced a significant increase in total distance travelled by 25.3% relative to control in the absence of CRF (Figure 2.6D; TCAP+CRF (0 µg): t = 4.004, p = 0.0003), although it had no effect with 1 µg CRF (t = 1.419, p = 0.170) or 3 µg CRF (t = 0.558, p = 0.582). Rearing, a vertical exploratory behaviour, was also measured, although TCAP-1 had no effect on these behaviours relative to controls (Table 2.2; TCAP+CRF (0 µg): t = 1.941, p = 0.0608; TCAP+CRF (1 µg): t = 0.151, p = 0.882; TCAP+CRF (3 µg): t = 0.149, p = 0.883).

2.4.2 Experiment 2: Effects of 5-day repeated TCAP-1 pre-treatment

Like Experiment 1, CRF was used as a stressor to distinguish TCAP-1’s effects on anxiety as determined by one-way ANOVA (Table 2.3). CRF significantly reduced percent open arm time in the EPM (F<sub>2,27</sub> = 4.321, p = 0.0235) whereas CRF tended to reduce percent open arm entries, although this trend did not reach significance (F<sub>2,27</sub> = 2.367, p = 0.113). In the OF, CRF had non-significant effects on centre entries, time, distance travelled, and total distance travelled (p > 0.05).
Table 2.3: Experiment 2: Acute injections of CRF in the EPM and OF.

<table>
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<tr>
<th></th>
<th>SAL+CRF (0 µg)</th>
<th>SAL+CRF (1 µg)</th>
<th>SAL+CRF (3 µg)</th>
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<td>EPM</td>
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<tr>
<td>% Open Arm Entries (%)</td>
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<td>% Open Arm Time (%) *</td>
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</tr>
<tr>
<td>Head-Dipping a</td>
<td>9.2</td>
<td>9.7</td>
<td>5.1</td>
</tr>
<tr>
<td>Stretched-Attend a</td>
<td>1.2</td>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>OF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centre Entries</td>
<td>211.7</td>
<td>103.2</td>
<td>180.4</td>
</tr>
<tr>
<td>Centre Time (s)</td>
<td>710.1</td>
<td>441.1</td>
<td>620.8</td>
</tr>
<tr>
<td>Centre Distance (cm)</td>
<td>3558.9</td>
<td>2238.8</td>
<td>3294.6</td>
</tr>
<tr>
<td>Total Distance (cm)</td>
<td>14460.0</td>
<td>8323.6</td>
<td>11606.9</td>
</tr>
</tbody>
</table>

*aExploratory behaviours could only be obtained from a portion of the group; SAL+CRF (0 µg): n = 5, SAL+CRF (1 µg): n = 6, SAL+CRF(3 µg): n = 7, (*p < 0.05).

Figure 2.7: Repeated TCAP-1 pre-treatment on CRF-induced behaviour in the EPM. Percent of control data for (A) percent open arm entries and (B) percent open arm time. Rats were pre-treated with 5 days of TCAP-1 and then given an i.c.v. challenge of 0, 1, or 3 µg CRF on test day. TCAP-1-treated groups are presented as a percentage of their respective saline-pre-treated controls. Percent open arm entries and percent open arm time were significantly reduced in the TCAP+CRF (1 µg) group relative to the SAL+CRF (1 µg) group, indicating that TCAP-1 potentiated the effects of 1 µg CRF. Bars represent mean percent of control ± SEM (*p < 0.05).
EPM data from the SAL+CRF (0 µg), SAL+CRF (1 µg), SAL+CRF (3 µg) groups (n = 10, 13, 7, respectively) and TCAP+CRF (0 µg), TCAP+CRF (1 µg), TCAP+CRF (3 µg) groups (n = 11, 8, 8, respectively) were obtained. TCAP-1 group data was converted to a percent of control for the corresponding saline group at each dose of CRF (0, 1, 3 µg). Percent open arm entries, percent open arm time, stretched-attend postures, head-dipping, and rearing were measured. TCAP-1 had no effect on percent open arm entries without CRF (Figure 2.7A; TCAP+CRF (0 µg): t = 1.006; p = 0.338) or with 3 µg CRF (TCAP+CRF (3 µg): t = 0.218, p = 0.834), however, TCAP-1 pre-treatment with a 1 µg CRF challenge had a significant effect reducing percent open arm entries by 39.4% relative to its saline-treated control (t = 4.986, p = 0.0016). Similarly, in percent open arm time, TCAP-1 pre-treatment had no effect without CRF or with a 3 µg injection of CRF on test day (Figure 2.7B; TCAP+CRF (0 µg): t = 1.059, p = 0.315; TCAP+CRF (3 µg): t = 0.674, p = 0.522), although TCAP-1 reduced percent open arm time by 37.6% under a 1 µg CRF challenge on test day (t = 2.718, p = 0.0299). In exploratory behaviours, TCAP-1 pre-treatment decreased rearing by 51.4% relative to its control when challenged with 3 µg CRF on test day (Table 2.4; t = 3.384, p = 0.0117) but had no effect at 0 µg or 1 µg doses of CRF (TCAP+CRF (0 µg): t = 0.916, p = 0.4019; TCAP+CRF (1 µg): t = 0.618, p = 0.599). TCAP-1 pre-treatment increased head-dipping by 63.0% in the absence of CRF relative to control (t = 4.493, p = 0.0064) but decreased head-dipping by 37.9% relative to controls when challenged with 1 µg CRF (Table 2.4; t = 6.351, p = 0.0239), but this effect was not seen in the TCAP-1 pre-treatment group that was challenged with 3 µg CRF (t = 0.938, p = 0.380). Finally, TCAP-1 pre-treatment had no effect on stretched-attend behaviours (Table 2.4) when challenged with either no CRF (t = 1.079, p = 0.330) or with 3 µg CRF (t = 1.210, p = 0.265), although TCAP-1 with 1 µg virtually eliminated stretched-attend postures in this experiment, relative to control.
Figure 2.8: Repeated TCAP-1 pre-treatment on CRF-induced behaviour in the OF. Percent of control data for (A) centre entries, (B) centre time, (C) centre distance travelled, and (D) total distance travelled. Rats treated with TCAP-1 and challenged with CRF (1, 3 µg) had significantly lower centre entries, time, distance, and total distance than saline pre-treated controls. Bars represent mean percent of control ± SEM (*p < 0.05, **p < 0.01).
Table 2.4: Experiment 2: Mean percent of control for exploratory behaviours in the EPM and OF.

<table>
<thead>
<tr>
<th></th>
<th>TCAP+CRF (0 µg)</th>
<th>TCAP+CRF (1 µg)</th>
<th>TCAP+CRF (3 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPM Rearing</td>
<td>118.6%</td>
<td>79.5%</td>
<td>48.6%*</td>
</tr>
<tr>
<td>Head-Dipping</td>
<td>163.0%**</td>
<td>62.1%*</td>
<td>72.9%</td>
</tr>
<tr>
<td>Stretched-Attend</td>
<td>55.6%</td>
<td>0.0%</td>
<td>145.8%</td>
</tr>
<tr>
<td>OF Rearing</td>
<td>108.2%</td>
<td>94.6%</td>
<td>44.5%**</td>
</tr>
</tbody>
</table>

Exploratory behaviours could only be obtained from a portion of the EPM group; SAL+CRF (0 µg): n = 5, SAL+CRF (1 µg): n = 6, SAL+CRF (3 µg): n = 7; TCAP+CRF (0 µg): n = 6, TCAP+CRF (1 µg): n = 3, TCAP+CRF (3 µg): n = 8, (*p < 0.05, **p < 0.01).

OF data from the SAL+CRF (0 µg), SAL+CRF (1 µg), SAL+CRF (3 µg) groups (n = 7, 6, 7, respectively) and the TCAP+CRF (0 µg), TCAP+CRF (1 µg), TCAP+CRF (3 µg) groups (n = 7, 7, 6, respectively) were obtained. Data from the first 10 min of the 60 min trial were analyzed, in order to facilitate comparisons with Experiment 1. TCAP-1 group data was converted to a percent of control for the corresponding saline group at each dose of CRF (0, 1, 3 µg), and the following measures were analyzed: centre entries, centre time, centre distance travelled, and total distance travelled. TCAP-1 had no effect on centre entries relative to saline-treated controls for CRF doses of 0 or 1 µg (Figure 2.8A; t = 0.0989, p = 0.3613; t = 1.550, p = 0.172, respectively), although pre-treatment of TCAP-1 with an acute challenge of 3 µg CRF reduced centre entries by 37.6% relative to its saline control (t = 3.419, p = 0.0189). TCAP-1 had no effect on centre time under unchallenged conditions or 3 µg CRF (Figure 2.8B; t = 0.656, p = 0.537; t = 2.036, p = 0.0973, respectively) although TCAP-1 as a pre-treatment had significant effects in rats challenged with 1 µg CRF (t = 2.853, p = 0.0291), decreasing the time spent in the centre of the OF by 43.3%. Similarly, in centre distance travelled, TCAP-1 had no effects in the absence of a challenge (Figure 2.8C; t = 1.300, p = 0.241), although it significantly reduced centre distance travelled in rats pre-treated with TCAP-1 and acutely challenged with 1 µg CRF (t = 2.550, p = 0.0435) and 3 µg CRF (t = 2.737, p = 0.0410) by 35.3% and 43.0%, respectively. TCAP-1 had no effects on total distance travelled at the 0 µg and 1 µg dose of CRF (Figure 2.8D; t = 0.586, p = 0.580; t = 0.345, p = 0.742, respectively) but TCAP-1 treatment reduced total distance in the 3 µg CRF group by 25.2% relative to its control (t = 4.637, p = 0.0056). Finally, TCAP-1 had no
effect on rearing on its own in the first 10 min of the trial, although it significantly reduced rearing in rats pre-treated with TCAP-1 and challenged with 3 µg of CRF (Table 2.4; t = 4.506, p = 0.0064).

Table 2.5: Experiment 2: Repeated TCAP-1 pre-treatment on CRF-induced behaviour in the OF for the full 60 minute trial as a mean percent of control

<table>
<thead>
<tr>
<th></th>
<th>TCAP+CRF (0 µg)</th>
<th>TCAP+CRF (1 µg)</th>
<th>TCAP+CRF (3 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centre Entries</td>
<td>94.3 ± 19.3 %</td>
<td>75.2 ± 18.4 %</td>
<td>50.5 ± 9.4 %**</td>
</tr>
<tr>
<td>Centre Time (s)</td>
<td>88.3 ± 21.4 %</td>
<td>47.6 ± 15.7 %*</td>
<td>49.0 ± 13.1 %*</td>
</tr>
<tr>
<td>Centre Distance (cm)</td>
<td>103.1 ± 24.1 %</td>
<td>61.1 ± 14.6 %*</td>
<td>55.2 ± 11.3 %*</td>
</tr>
<tr>
<td>Total Distance (cm)</td>
<td>100.0 ± 11.0 %</td>
<td>110.1 ± 10.6 %</td>
<td>97.2 ± 30.9 %</td>
</tr>
</tbody>
</table>

(*p < 0.05, **p < 0.01)

OF data were also analyzed for the full 60 min trial, producing results that were similar to the 10 min test (Table 2.5). TCAP-1 pre-treatment with an acute 3 µg CRF dose produced the most consistent effects, reducing centre entries by 49.5%, centre time by 51.0%, and distance travelled by 44.8%, and relative to controls (p = 0.0033, p = 0.0114, p = 0.0107, respectively). TCAP-1 pre-treatment with an acute dose of 1 µg CRF decreased centre time by 52.4% and decreased centre distance by 38.9% relative to saline-treated controls (p = 0.0156, p = 0.0371, respectively).

2.4.3 Experiment 3: Effects of 10-day repeated TCAP-1 treatment

In contrast to Experiment 1 and 2, restraint was used as a long-term stressor in this experiment (Table 2.6). Restraint produced a tendency to increase percent open arm entries and time, but this was not significant using a one-way ANOVA (p > 0.05).

Table 2.6: Experiment 3: Restraint as a stressor in the EPM.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Open Arm Entries (%)</td>
<td>62.5</td>
<td>74.6</td>
</tr>
<tr>
<td>% Open Arm Time (%)</td>
<td>48.3</td>
<td>62.3</td>
</tr>
<tr>
<td>Rearing</td>
<td>12.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Head-Dipping</td>
<td>9.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Stretched-Attend</td>
<td>7.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Figure 2.9: Repeated TCAP-1 on restraint-induced behaviour in the EPM. Percent of control data for (A) percent open arm entries and (B) percent open arm time. The TCAP-1 treatment group had a significantly higher mean percent open arm time than its saline-treated group, whereas the TCAP-1 treatment group subjected to daily restraint had a significantly lower mean percent open arm time relative to its saline-treated group. Bars represent mean percent of control ± SEM (*p < 0.05).

For the EPM, data were obtained from SAL (n = 4), TCAP (n = 5), SAL+Restraint (n = 5), and TCAP+Restraint (n = 4) groups. TCAP-1 group data were converted to a percent of control compared to the mean of the corresponding saline-treated group without or with restraint. Several measures were investigated: percent open arm entries, percent arm time, stretched-attend postures, head-dipping, and rearing. In percent open arm entries (Figure 2.9A), TCAP-1 with restraint had no significant effect relative to its saline-treated controls (t = 1.971, p = 0.143), and TCAP-1 in the absence of restraint may have increased percent open arm entries, but this effect was just below statistical significance (t = 2.624, p = 0.0585). TCAP-1 had effects on the percent open arm time in the absence of restraint, increasing the percent open arm time by 24.9% relative to control (Figure 2.9B; t = 4.191, p = 0.0138), and decreasing the percent open arm time in restraint groups by 11.5% relative to its control (t = 3.373, p = 0.0433). TCAP-1 had no effects on rearing, without or with restraint (Table 2.7; t = 0.923, p = 0.409; t = 0.951, p = 0.412, respectively), nor did TCAP-1 have effects on head-dipping (Table 2.7; TCAP: t = 1.432, p = 0.225; TCAP+Restraint: t = 1.725, p = 0.183). However, TCAP-1 did have effects on stretched-attend postures, a type of risk-assessment behaviour. TCAP-1 treatment, in the absence of restraint, decreased stretched-attend postures by 25.7% relative to control (Table 2.7; t = 3.087, p
but this effect was not seen in the TCAP+Restraint group relative to its restraint control (t = 1.984, p = 0.142). There were no differences in any of the groups in total distance travelled in the maze (data not shown).

Table 2.7: Experiment 3: Mean percent of control for exploratory behaviours in the EPM

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>TCAP+No Restraint</th>
<th>TCAP+Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rearing</td>
<td>75.30%</td>
<td>113.20%</td>
</tr>
<tr>
<td>Head-Dipping</td>
<td>80.00%</td>
<td>66.86%</td>
</tr>
<tr>
<td>Stretched-Attend</td>
<td>74.29%*</td>
<td>77.40%</td>
</tr>
</tbody>
</table>

(*p < 0.05)

2.5 Discussion

The experiments described in this chapter were designed to explore the effects of acute or repeated i.c.v. TCAP-1 on stress-related behaviours in two unconditioned tests of anxiety, the EPM and OF tests. TCAP-1 administered acutely did not have robust effects on behaviour with or without CRF co-treatment. In contrast, 5 days of TCAP-1 pre-treatment significantly potentiated CRF-induced behaviour in both the EPM and OF tests, but did not have robust effects in the absence of a stressor. Finally, 10 days of TCAP-1 treatment affected behaviours in the EPM both in the presence and absence of a restraint stressor. These sometimes contrasting results indicate that TCAP-1 may be acting as a behavioural modulator, which is supported by previous work (Wang et al., 2005), which showed that acute injections of TCAP-1 into the basolateral nucleus of the amygdala of rats had differential effects on the acoustic startle response depending upon the rat’s baseline reactivity. Thus, these studies confirmed that TCAP-1 administration is capable of modulating stress-associated behaviour and may act in part by regulating the CRF system.

The EPM test has been used extensively as a test of anxiety (Carobrez and Bertoglio, 2005; Pellow et al., 1985; Pellow and File, 1986; Rodgers and Dalvi, 1997). The maze has been validated using known anxiolytics that have efficacy in humans, such as chlordiazepoxide and diazepam (Pellow et al., 1985), which increase the percentage of entries into, and time spent on the open arms of the maze. Drugs that increase anxiety in humans, such as amphetamine or caffeine, reduce these parameters (Pellow et al., 1985). However, the EPM may not predict
anxiety-modulating measures in all substances, as it does not predict the anxiolytic-like properties of antidepressants, such as selective serotonin reuptake inhibitors, which are used in the treatment of some anxiety disorders (Borsini et al., 2002). Exposure to the open arms of the maze increases serum corticosterone levels (Pellow et al., 1985), as the aversion to open spaces, but not the height or novelty of the maze, produces a stressful environment (Treit et al., 1993). Supplementary exploratory behaviours in the EPM, such as rearing, head-dipping, and stretched-attend postures, have been used as additional measures that have ethological validity but may be more sensitive to anxiety-modulating drugs than traditional open arm measures (Carobrez and Bertoglio, 2005). Rearing is a type of vertical exploratory activity (Ohl, 2003), head-dipping is classified as directed exploration beneath the maze, and the stretched-attend posture is a defensive posture and risk assessment behaviour (Weiss et al., 1998). In particular, risk assessment behaviours may be separate from anxiety, and may persist even in animals that readily venture into unprotected spaces (Ohl, 2003). Manipulations that increase anxiety tend to decrease rearing (Rodgers and Dalvi, 1997) and increase head-dipping and stretched-attend postures (Kaesermann, 1986; Mikics et al., 2005; Rodgers and Cole, 1993).

The OF test is similar to the EPM test in that it measures the time, entries, and distance travelled in the open space of a novel arena (i.e. the centre), away from tactile stimuli such as inescapable walls (Lister, 1990). Rats will generally avoid the centre portion of the chamber, and anxiolytic manipulations will increase the time spent in the centre of the arena (Crawley, 1985; Prut and Belzung, 2003). Rearing, a type of exploratory behaviour, was also measured; anxiety-inducing treatments, such as i.c.v. injection of CRF, decreases rearing behaviour (Britton et al., 1984; Sherman and Kalin 1986; Sutton et al., 1982). Although not as widely used as the EPM, the OF can be used to elucidate effects on locomotion to ensure that anxiolytic effects seen in either test are not confounded simply by an alteration in locomotion.

Two different types of stimuli, acute CRF and repeated restraint, were used in these studies to induce stress-like conditions. Acute CRF produced anxiogenic effects in Experiments 1 and 2, similar to previous studies (Adamec et al., 1991; Adamec and McKay, 1993; File et al., 1988; Sutton et al., 1982). In contrast, repeated restraint produced a tendency, although not significant, to increase percent open arm entries and time. It may seem paradoxical that repeated stressors may decrease anxiety; however, as displayed in Experiment 3, organisms can habituate to the same (i.e. homotypic) repeated stressor, as repeated restraint had little, if any, effect on the EPM.
For example, 7 days of restraint (2 h per day) produced anxiolytic effects in the light-dark box (Cancela et al., 1995) although a single restraint session produced anxiogenic effects. Similarly, 9-10 days of restraint (1 h per day) had no effect on EPM behaviour although a single restraint session increased anxiety in the EPM test (Thorsell et al., 1999), and 8 days of restraint (30 min per day) reduced anxiety in the EPM compared to acute restraint (Jaferi and Bhatnagar, 2007). Thus, a small amount of predictable stressors may be beneficial to the organism. Habituation of the stress response occurs at an endocrine level, as a single restraint session significantly increases corticosterone and ACTH levels, but multiple restraint exposures decrease corticosterone and ACTH (Culman et al., 1991; Dhabhar et al., 1997; File, 1982). However, although the effects of repeated restraint on endocrine and behavioural measures may appear attenuated, multiple exposures to restraint still have lasting effects in the brain, as evidenced by atrophy of the dendrites of hippocampal cells and hypertrophy of the dendrites in amygdala cells (see Chapter 4).

Acutely, TCAP-1 did not have any robust behavioural effects with or without a co-injection of CRF. TCAP-1 increased rearing exploratory behaviours in the EPM and increased total distance travelled in the OF in the absence of CRF relative to controls. Exploratory behaviours may be used as another stress-like behavioural dimension but does not replace the traditional open arm measures; therefore, although rearing behaviours are decreased under stressful conditions, we cannot conclude that this treatment of TCAP-1 decreased anxiety per se. Similarly, an increase in distance moved in TCAP-1-treated populations, although not necessarily an indication of anxiety, may indicate an increase in exploratory behaviour in a novel environment. This is in contrast to the effects of CRF in this experiment, which decreased total locomotion. The doses of CRF used in this experiment are relatively high (1 µg CRF is equivalent to 0.21 nmol, whereas 3 µg CRF is equivalent to 0.63 nmol), and high doses (0.15 nmol) of i.c.v. CRF decrease locomotion and rearing in a novel open field (Sutton et al., 1982). However, the effects of CRF exhibit an inverse-U shaped curve: low doses (0.0015 nmol) increase locomotion and rearing, and evoke a “behaviourally activating” or “hypervigilance” response similar to behaviour elicited after non-traumatic mild stress such as white noise or bright light (Dunn and Berridge, 1990; Roth and Katz, 1979). TCAP-1, therefore, may be inducing an acutely “behaviourally active” state which may manifest itself by an increase of activity and exploratory
behaviour in order to actively obtain information about a potential threat (Ohl, 2003), but this behaviour is eliminated in the presence of more stressful stimuli.

The results of acute injection of TCAP-1 were not as pronounced as expected, given that a sister-study that investigated c-Fos protein induction (see Chapter 3) provided intriguing and robust results. Previously, acute injections of TCAP-1 into the amygdala had modulatory effects in the absence of an additional stressor in the acoustic startle test (Wang et al., 2005). However, it is possible that the time course used in this study, which is identical to the time course used in the c-Fos induction experiment, was not appropriate to see behavioural modifications to CRF-induced exploratory behaviour. The anxiogenic effects of CRF are still present 2 h after i.c.v. administration (Spina et al., 2002), but other peptides may have shorter windows with which to see effects. For example, low doses of neuropeptide Y (NPY) in mice have anxiogenic effects on the EPM 10 min after injection but these effects are not detected 30 min post-injection (Nakajima et al., 1998), which may be a result of a biphasic disappearance curve with relatively short half-lives (4.1 and 20 min) (Pernow et al., 1987). Similarly, TCAP-1 has a relatively short half-life of roughly 3.7-15.8 min in blood when given intravenously (Song, Barsyte, and Lovejoy, manuscript in preparation), suggesting that although 2 h was sufficient to see the effects of TCAP-1 on CRF-induced c-Fos induction, the behavioural effects of reduced c-Fos in specific areas of the brain may have already subsided prior to behavioural testing.

Repeated injections of TCAP-1 had differential effects, depending on the treatment regimen. The first group of experiments consisted of 5 days of daily TCAP-1 injections (Experiment 2) followed by a 1-week rest period before CRF challenge and behavioural testing, which I have referred to as “pre-treatment”. This treatment paradigm was used previously (Wang et al., 2005) to demonstrate long-term effects of TCAP-1 in the acoustic startle test. This regimen is similar to the “sensitization” protocol seen in stress literature demonstrating that multiple exposures to a single type of stressor, for example, CRF, swim stress, or drugs of abuse, potentiates the effects of a “challenge”, such as amphetamine, cocaine, or a heterotypic stressor, after a stress-free period (Cador et al., 1993; Cole et al., 1990; Dronjak et al., 2004; Piazza et al., 1990; Schmidt et al., 1999; Stewart and Badiani, 1993). Therefore, the sensitization paradigm was utilized to potentially enhance the effect of repeated injections of TCAP-1 with a challenge of i.c.v. CRF.
In the EPM, pre-treatment with TCAP-1 without an added stress challenge did not affect open arm measures, although it significantly increased head-dipping, a type of exploratory behaviour. This increase in directed exploration is reminiscent of the results seen in the acute studies, although in the TCAP-1 pre-treatment experiment, testing was performed one week after the last TCAP-1 treatment; long-term changes would have to occur that were facilitated by multiple exposures to TCAP-1. Exposure to the EPM produces a significant rise in plasma corticosterone (Rodgers et al., 1999) and is generally regarded as a mild stressor. Therefore, like the acute study, TCAP-1 could be potentiating the effect of a mild stress: in this case, facilitating “behavioural activation” (Sutton et al., 1982) and increasing directed exploration. In the two stress conditions, pre-treatment with TCAP-1 potentiated the effect of the CRF challenge. Rats treated with 5 days of TCAP-1 showed decreased percent open arm time and percent open arm entries in response to a 1 µg CRF challenge relative to rats treated with saline and CRF. This treatment also decreased head-dipping and eliminated stretched-attend postures relative to controls. TCAP-1 pre-treatment with a higher dose of CRF (3 µg) did not increase these stress-potentiating effects in the EPM, although it did decrease rearing relative to controls. The absence of an effect may be due to an unexpected blunted effect of the higher dose of CRF, as 3 µg did not appear to be as severe a stressor as the 1 µg dose, although I have shown that 3 µg of CRF indeed does have anxiogenic effects (Experiment 1). However, TCAP-1 did decrease rearing when challenged with 3 µg CRF compared to controls, which suggests that TCAP-1 potentiated the effects of CRF at this dose.

In the OF test, rats were tested for 60 min and analyzed to record data for both the full 60 min session as well as the first 10 min of the session in order to compare data with Experiment 1. The 60 min test investigated whether TCAP-1 altered behaviour after the novelty of the OF subsided, but in general the results were similar to the first 10 min of the trial. Pre-treatment of rats with 5 days of i.c.v. TCAP-1 had no effect without an added stressor challenge, as TCAP-1 pre-treated rats were virtually indistinguishable from their saline pre-treated controls. Interestingly, TCAP-1 treatment did not increase total locomotion, either in the first 10 min or for the whole 60 min trial, unlike the results from Experiment 1. In the first 10 min, pre-treatment of TCAP-1 potentiated the effects of both 1 µg and 3 µg CRF, as TCAP-1 pre-treatment decreased centre time and centre distance in rats challenged with 1 µg CRF relative to controls, whereas TCAP-1 pre-treatment decreased centre entries, centre distance, total distance
travelled and rearing in rats challenged with 3 µg CRF relative to controls. Data from the whole 60 min were similar to the results from the first 10 min, with the exception that after 60 min, TCAP-1 did not decrease total distance moved in rats treated with 3 µg CRF relative to control. The results from the OF test suggest that TCAP-1 pre-treatment, although it does not itself alter behaviour in the test, it did potentiate the effects of both 1 and 3 µg of CRF.

Taken together, the results of the EPM and OF tests in TCAP-1 pre-treated populations indicate that TCAP-1 by itself does not affect traditional measures in either test, but may increase exploratory behaviour such as head-dipping. However, under an acute challenge such as an i.c.v. injection of CRF, TCAP-1 pre-treatment increases the effect of the stressor and appears to be increasing anxiety-like behaviour.

The second repeated treatment paradigm (Experiment 3) consisted of 10 days of i.c.v. TCAP-1 injections with EPM testing performed 24 h after the last injection. During the 10-day injection period, rats were subjected to 2 h per day of restraint in a clear Plexiglas tube in their home cages. Restraint was used as a psychological stressor that utilizes limbic system integration before activation of the HPA axis and behavioural responses. Rats from these behavioural tests were used to investigate dendritic spines in the hippocampus and amygdala (see Chapter 4), and thus the treatment paradigm with the concurrent restraint sessions followed a timeline similar to the Vyas et al. (2002) study that produced dendritic remodelling. The premise of this experiment was that rats will respond to the repeated stressor by altering levels of catecholamines, ACTH, and hormones such as prolactin (Culman et al., 1991; Kant et al., 1983; McCarty et al., 1988), increasing basal corticosterone levels (Dronjak et al., 2004), blunting the corticosterone response to the homotypic stressor (Kim and Han, 2006; Ma et al., 1999), potentiating the corticosterone response to a heterotypic stressor (Dronjak et al., 2004; Ma et al., 1999), and inducing anxiety-like behaviour (Chotiwat and Harris, 2006; Suvrathan et al., 2010; Vyas et al., 2002). However, if TCAP-1 affects behaviour primarily in the presence of a stressor, we hypothesized that daily TCAP-1 injections a few hours prior to daily restraint may alter the effect of the stressor on the rat, modulating both neuronal morphology (see Chapter 4) and behaviour.

In the EPM, 10 days of TCAP-1 increased percent open arm time in the absence of a stressor, which is in contrast to other experiments that have used the EPM (Al Chawaf et al., 2007b; Tan et al., 2008, 2009) where TCAP-1 treatment under basal conditions did not affect open arm
measures. This may be a result of the number of doses given under this treatment regimen, as long-term exposure to TCAP-1 may be required to elicit effects in the absence of a challenge. Ten days of TCAP-1 administration also decreased stretched-attend postures. Taken together these two measures suggest that long-term i.c.v. administration of TCAP produces anxiolytic effects. In restrained animals, TCAP-1 treatment decreased percent open arm time relative to saline-treated controls. However, it should be noted that the restraint stress did not induce anxiety-like behaviour in saline-treated rats, and therefore we cannot conclude that TCAP-1 potentiated the effect of restraint as it did in Experiment 2. As previously discussed above, repeated restraint induced a trend towards decreased anxiety, which is in disagreement with numerous previous reports (Gameiro et al., 2006; Sevgi et al., 2006; Suvrathan et al., 2010; Vyas et al., 2002), but in agreement with others (Cancela et al., 1995; Jaferi and Bhatnagar, 2007). This discrepancy can possibly be explained by the severity of the stressor used in the experiment. For example, the Chattarji group (Suvrathan et al., 2010; Vyas et al., 2002) utilized complete immobilization in a “Decapicone”, which is a more severe form of restraint than the Plexiglas restraint tube, which still allows a small, but noticeable, amount of movement. The restraint in which we exposed our rats may be described as both predictable and mild, as our rats continually entered restraint tubes each day without a struggle. The predictability and low severity of the stressor may be beneficial, as chronic predictable restraint stress decreased anxiety in the EPM and novel object recognition test (Parihar et al., 2011). The authors postulate that predictable chronic stress produces a lower level of corticosterone output from the HPA axis, which has pro-cognitive and anxiety-reducing effects. Therefore, as TCAP-1 had decreased percent open arm time relative to saline controls, TCAP-1 may indeed be eliciting increased anxiety in the presence of a mild stressor without potentiating the effect of the stressor.

Although these behavioural studies indicate that TCAP-1 has the capability to regulate CRF-dependent behaviours, the mechanism is not clear. A previous study indicated that TCAP-1 does not interact with either CRF receptor directly (Wang et al., 2005), although this does not discount the possibility that TCAP-1 may be involved in downstream signal transduction systems in the cell. For example, TCAP-1 modulates cAMP accumulation in Gn11 immortalized hypothalamic neurons independently of CRF receptor binding (Wang et al., 2005), and in high concentrations, TCAP-1 may decrease cAMP accumulation without interacting directly with the CRF receptors, attenuating the cell’s response to CRF. TCAP-1 may also interfere with regulatory proteins,
such as transcription factors that are involved in genomic responses to CRF. For example, immediate-early gene proteins such as Fos and Jun are highly upregulated after exposure to stressors, such as restraint or CRF injection. Fos, for example, is upregulated via increases in cAMP which causes a phosphorylation of cAMP response element binding protein (CREB). Immediate-early gene proteins then dimerize and bind to activator protein-1 (AP-1) sites which then produce a host of effects, such as increased transcription of brain-derived neurotrophic factor (BDNF), which increases synaptic plasticity, and increased trafficking of CRF receptors, which could increase sensitivity to additional stress insults.

Together, these studies indicate that TCAP-1 has differential effects based on the severity of the stressor. Under low-stress conditions (CRF (0 µg) and no restraint), TCAP-1 increased exploratory behaviours and decreased risk assessment, and appeared to decrease the anxiety state. In contrast, under high-stress conditions (CRF (1 µg), CRF (3 µg), or restraint), TCAP-1 appeared to increase the anxiety state. It is possible that TCAP-1 may affect stress along CRF’s inverse-U shaped curve; TCAP-1 could be enhancing the effect of the low-stress experienced in novel environments such as the EPM and OF by increasing “behavioural activation” (Sutton et al., 1982) and enhancing the effect of high-stress elicited by exogenous stressors, thereby increasing the “anxiety state”. In summary, I have shown that i.c.v. TCAP-1 does exert behavioural effects in exploratory tests of anxiety, and that TCAP-1 enhances the effects of low and high levels of stress.

2.6 References


3 Chapter Three: Elucidating TCAP-1 responsive areas of the brain

Part of this chapter has been published in a modified form in:


3.1 Abstract

The teneurin C-terminal associated peptides (TCAPs) are a novel family of four endogenous peptides that have shown bioactive properties both in vitro and in vivo. Previously, I and others have shown that both acute and repeated intracerebral injections of synthetic TCAP-1 modulate anxiety-like behaviours in male rats in tests of anxiety, although the neural substrates responsible for these effects were unknown. In the current study, I examined the induction of the immediate-early gene, c-Fos, after acute or repeated intracerebral injections of TCAP-1 into male rats, alone or in concert with CRF. The results indicate that whereas an acute injection of TCAP-1 did not induce c-Fos on its own, an acute TCAP-1 injection attenuated the CRF-induced increase in c-Fos expression in the limbic system and many of the areas associated with the behavioural responses to stress, including the hippocampus, central and basolateral nuclei of the amygdala, medial prefrontal cortex, and dorsal raphe nucleus. Other areas, such as the paraventricular nucleus of the hypothalamus, bed nucleus of the stria terminalis, medial nucleus of the amygdala, and locus coeruleus, displayed CRF-induced c-Fos levels that were unaffected by TCAP-1. However, when TCAP-1 was injected daily for five days as a pre-treatment regimen, TCAP-1 did not affect basal levels of c-Fos, nor did it attenuate the effects of a CRF challenge in any of the areas measured, including the areas that had been attenuated in the acute study. In both the acute and repeated TCAP-1 treatment experiments, there was no difference in the coefficient of variation between TCAP-1 and the saline treated groups, however, the coefficient of variation in the TCAP-1 + CRF treated groups was significantly greater than groups treated with CRF in both experiments. This indicates that whereas the acute TCAP-1 can attenuate CRF in certain areas of the brain, but repeated TCAP-1 does not, either treatment regimen of TCAP-1 can increase the
variability of c-Fos responses under stress; this suggests that like previous behavioural experiments, individual differences may play an important role in responses to TCAP-1.

### 3.2 Introduction

TCAP-1 is a novel biologically active peptide that *in vitro* can modulate cAMP accumulation and cell proliferation (Qian et al., 2004) and, when injected into the basolateral nucleus of the amygdala, can modulate acoustic startle behaviour (Wang et al., 2005). Previous work had indicated that TCAP-1 mRNA is found in areas of the brain associated with the regulation of behavioural adaptations to stress, such as the hippocampus, amygdala, and hypothalamus (Wang et al., 2005). Furthermore, TCAP-1 has behavioural effects in animal approach-avoidance conflict tests, such as the EPM and open field tests (see Chapter 2; Tan et al., 2008). Therefore, it was imperative to elucidate the areas of the brain sensitive to TCAP-1 under both acute treatment and repeated pre-treatment regimens, as these treatment regimens were successful in eliciting behavioural effects (Tan et al., 2008; Wang et al., 2005).

Although the expression of TCAP-1 was located in the limbic system and throughout the brain, peptide localization does not necessarily translate to peptide action. For example, the lateral central nucleus of the amygdala is strongly immunoreactive for CRF but is relatively devoid of CRF receptor expression (Bittencourt and Sawchenko, 2000; Justice et al., 2008). Therefore, I used the immediate-early gene protein, c-Fos, which is a transcription factor of the activator protein-1 (AP-1) family, as a measure of general neuronal activation in the brain. AP-1 proteins can form heterodimers between the Jun proteins (c-Jun, Jun B, and Jun D) and the Fos proteins (c-Fos, Fos B, Fra 1, Fra 2) to form the AP-1 complex, which binds to the AP-1 response element to activate the transcription of genes that regulate neuronal proliferation, apoptosis, and differentiation (Herdegen and Waetzig, 2001). The c-Fos protein has a low basal expression but is highly inducible, as c-Fos is increased in neurons in response to a variety of stimuli such as stress, learning, and nociception (Herrera and Robertson, 1996). C-Fos mRNA is increased within a few minutes of the stimuli (Imaki et al., 1995), and the protein shows its maximal signal 2 hours after induction, with c-Fos returning to basal levels after 4 hours (Bittencourt and Sawchenko, 2000; Imaki et al., 1993), which makes c-Fos an ideal marker for neuronal activation.
CRF acts as a neurotransmitter/neuromodulator outside of the HPA axis, and the wide distribution of CRF$_1$ receptors in areas of processing and integration of sensory information (van Pett et al., 2000) allows for modulation of adaptive behaviours in response to stress. As highlighted in Chapter 2, i.c.v. CRF can be used to elicit many of the behavioural and physiological manifestations that are likewise elicited by stressors such as restraint, forced swim, and immune challenge (Koob et al., 1993; Korte and De Boer, 2003). I.c.v. CRF can dose-dependently increase c-Fos in the brain (Bittencourt and Sawchenko, 2000) in patterns similar to stressors such as restraint, footshock, forced swim, and immune challenge (Crane et al., 2005; Cullinan et al., 1995; Imaki et al., 1993; Rivest and Laflamme, 1995). C-Fos induction has also been used to gauge long-term changes in responses to stress, such as habituation, where multiple administrations of a homotypic stressor results in an attenuated stress-induced c-Fos response (Girotti et al., 2006; Melia et al., 1994; Watanabe et al., 1994) and sensitization, where a heterotypic stressor results in a potentiated stress-induced c-Fos response (Armario et al., 2004; Melia et al., 1994;). Therefore, i.c.v. CRF has been used as a predictable and dose-dependent stressor, as protocols for administering stressors such as restraint, swim stress, and footshock can vary between laboratories.

The mechanism by which TCAP-1 regulates CRF-mediated behaviour is not known. Therefore, these experiments determined the areas of the brain that are sensitive to TCAP-1, and examined potential interactions between TCAP-1 and CRF in the brain. I.c.v.-administered CRF was used in the current study as a positive control, and also to assess the effects of i.c.v.-administered TCAP-1 on CRF-induced c-Fos immunoreactivity. Therefore, I treated rats with TCAP-1 and CRF to determine if TCAP-1 could modulate the expected CRF-induced c-Fos accumulation.

### 3.3 Materials and Methods

#### 3.3.1 Animals

Male Wistar rats (n = 80; acute study, n = 40, repeated study, n= 40) weighing 250-275 g were obtained from Charles River Laboratories (Montreal, QC). Rats were singly housed in Plexiglas shoebox cages under standard laboratory conditions (12:12 h light:dark cycle, lights on at 0700 h, temperature 21 ± 1 °C) with food and water available ad libitum. Rats were given one week to acclimatize to laboratory conditions before surgery. All procedures were approved by the
University of Toronto Animal Care Committee in accordance with the Canadian Council on Animal Care.

3.3.2 Surgery

Male Wistar rats were anesthetized with isoflurane (3% induction, 2–3% maintenance in 100% O₂) and fit into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Analgesics (0.5 mg/kg buprenorphine for acute administration animals; 2.5 mg/kg ketoprofen for repeated administration animals) were administered subcutaneously at the beginning of surgery. A midline incision was made along the top of the head, exposing bregma. A 22-gauge guide cannula (Plastics One, Roanoke, VA, USA) was implanted into the right lateral ventricle using the following flat-skull coordinates: AP −1.0 and ML −1.4 from bregma, DV −2.7 from dura (Paxinos and Watson, 1998). The guide cannula was secured to the skull using four jewellers’ screws and dental cement. The opening of the cannula was covered by a removable cannula dummy. The wound was treated with Hibitane (Pfizer Animal Health, Kirkland, QC) to prevent infection. Animals were kept under warm lamps until regaining consciousness and provided with wet chow fortified with Nutri-Cal (Vetoquinol USA, Fort Worth, TX) in a clean cage for 12 h. Rats were then transferred to a clean cage and were given one week to recover from surgery.

3.3.3 Peptides

Synthetic mouse TCAP-1 was synthesized at 95% purity using f-moc-based solid phase synthesis (American Peptide Company, Sunnyvale, CA). For the acute TCAP-1 administration, TCAP-1 stocks were prepared to a concentration of 2.1 × 10⁻⁴ M (equivalent to 1 μg/μl TCAP-1) by treating lyophilized TCAP-1 with ammonium hydroxide vapour, then dissolving in sterile saline. The TCAP-1 dose was based on the molar equivalent of 3 μg of CRF. For the repeated TCAP-1 administration, TCAP-1 stocks were prepared to a concentration of 10⁻⁴ M. CRF (Sigma–Aldrich, Oakville, ON) was dissolved in sterile saline at a concentration of 1 μg/μl.

3.3.4 Experiment 1: Acute effects of TCAP-1 on c-Fos immunoreactivity

3.3.4.1 Injections

Injections were given between 0800 h and 1000 h. I.c.v. injections were delivered at a rate of 2 μl/min by a Hamilton syringe connected to a pump (Razel Scientific Instrument Inc., Stamford, CT) and connected via PE-50 tubing to a 28-gauge stainless steel injector that extended 1 mm
below the cannula guide tip. Animals were injected first with either 3 μl of sterile saline (vehicle) or 3 μg of TCAP-1. The injector was left in place for 60 s following infusion to prevent backflow up the cannula. Afterwards, the injector was removed, and a second injector was inserted into the cannula. An infusion of either 1 μl of saline or 1 μl of 1 μg/μl CRF was injected. There were four treatment groups, each with a total volume of 4 μl: saline + saline (“SAL”), saline + CRF (“CRF”), TCAP-1 + saline (“TCAP”), and TCAP-1 + CRF (“TCAP + CRF”).

3.3.4.2 Tissue processing

After injections, rats were returned to their home cages and the colony for 2 h. After that period, rats were removed from the colony and overdosed with isoflurane and decapitated. The brains were removed within 90 s, rinsed in PBS, and snap-frozen in −70 °C isopentane. The brains were then stored at −80 °C until they could be processed. Brains were mounted to the cryostat pedestal with Tissue-Tek OCT media (Sakura Finetek, Torrance, CA) and brains were sectioned on the coronal plane in a 1-in-6 series on a cryostat at a thickness of 25 μm. Sections were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Canada) and allowed to dry overnight before being stored at −80 °C until processing.

3.3.4.3 Immunohistochemistry

Immunohistochemical protocols were adapted from Sundquist and Nisenbaum (2005). All wash steps were performed with Tris-buffered saline with Tween-20 (TBS-T; 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6). Antibodies were diluted in TBS-T with 1% weight by volume IgG-free bovine serum albumin fraction V (BSA; Jackson ImmunoResearch, Burlington, ON). Slides were immersed in 4% phosphate-buffered paraformaldehyde (PFA) for 10 min. Endogenous peroxidases were quenched with a 1% hydrogen peroxide/methanol solution for 15 min. Endogenous proteins were blocked with 10% normal goat serum (Vector Laboratories, Burlington, ON). The sections were incubated with rabbit anti-c-Fos IgG (1:2000; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h in a standard humid chamber, then incubated in biotinylated goat anti-rabbit IgG (1:200; BA-1000, Vector Laboratories) for 30 min. The sections were reacted with an avidin–biotin–peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories) for 30 min according to kit instructions. Subsequently, the sections were visualized using diaminobenzidine chromagen (DAB Substrate Kit, Vector Laboratories). The sections were dehydrated using graded ethanol, cleared in xylene, and coverslipped with
Permount and coverglass (Fisher Scientific, Ottawa, ON). An adjoining series of sections were Nissl-stained by dehydrating the sections in graded ethanols, defatting in xylene, rehydration in graded ethanols and staining in 0.1% cresyl violet. Slides were then dehydrated in ethanol, cleared in xylene, and coverslipped with Permount and coverglass.

3.3.4.4 Analysis

Areas of the brain that are associated with the stress response and demonstrate high c-Fos activity in response to CRF were investigated. This included components of the limbic system (amygdala and hippocampus) and other areas linked to both the limbic system and the regulation of stress, including the medial prefrontal cortex (infralimbic and prelimbic cortices), paraventricular nucleus of the hypothalamus, septum (medial and lateral), bed nucleus of the stria terminalis, locus coeruleus, and the dorsal raphe nucleus.

Sections were scanned for c-Fos activity using a Nikon Optiphot microscope (Nikon Canada, Mississauga, ON). C-Fos-positive cells were manually counted for each area using NIS Elements Software Basic v2.10 (Nikon Canada, Mississauga, ON). Cumulative counts through the entire series for each region of interest were obtained for each brain. The observer was blind to the study.

3.3.5 Experiment 2: Repeated TCAP-1 pre-treatment on c-Fos immunoreactivity

3.3.5.1 Injections

Injections were given between 0800 h and 1000 h. I.c.v. injections were delivered as indicated above. The rats were injected once daily for five days with either 3 µl sterile saline (vehicle) or 3 µl of 10⁻⁴ M TCAP-1 and then returned to their cages. Rats were then allowed to rest for 7 days. On test day, rats were injected (i.c.v.) with 1 µl of saline (vehicle) or 1 µg/µl CRF and then returned to the colony. There were four treatment groups: saline pre-treatment with acute saline challenge (“SAL+SAL”), saline pre-treatment with acute CRF challenge (“SAL+CRF”), TCAP-1 pre-treatment with acute saline challenge (“TCAP+SAL”), and TCAP-1 pre-treatment with acute CRF challenge (“TCAP+CRF”). Rats were sacrificed 2 h after CRF injection and their brains were excised and snap-frozen in cold isopentane. Brains were mounted to the cryostat pedestal with Tissue-Tek OCT media (Sakura Finetek, Torrance, CA) and brains were
sectioned on the coronal plane in a 1-in-6 series on a cryostat at a thickness of 25 µm and thaw-mounted on SuperFrost Plus slides and stored at -80 °C until immunohistochemical preparation.

3.3.5.2 Immunohistochemistry

Owing to antibody lots from Santa Cruz Biotechnologies with vastly different efficacy, a modified immunohistochemistry protocol was adapted from Experiment 1. Immunohistochemical protocols were adapted from Sundquist and Nisenbaum (2005). All wash steps were performed with TBS-T. Antibodies were diluted in TBS-T with 1% weight by volume IgG-free BSA. Sections were immersed in 4% phosphate-buffered PFA for 10 minutes. Exogenous peroxidases were quenched with a 1% hydrogen peroxide/methanol solution for 15 min. Sections were then blocked with an avidin-biotin block (Vector Laboratories) at half-strength for 15 min. Endogenous proteins were blocked with 10% normal goat serum and then incubated with rabbit anti-c-Fos IgG (1:1000, Santa Cruz Biotechnology) for 24 h at room temperature in a standard humid chamber. Sections were then incubated in biotinylated goat anti-rabbit IgG (1:200) for 30 mins, reacted with the ABC complex for 30 min, and then visualized with DAB. The sections were dehydrated with alcohol, cleared in xylene, and coverslipped with Permount and coverglass. An adjoining series of sections was stained using cresyl violet for reference purposes.

3.3.5.3 Analysis

Areas of the brain that were quantified in Experiment 1 were also quantified in Experiment 2, with the inclusion of olfactory areas on the ventral side of the brain. Sections were scanned for c-Fos-positive cells as detailed in Experiment 1. The observer was blind to the treatment groups.

3.3.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.0. Data was analyzed using a two-way ANOVA with “TCAP-1” and “CRF” as factors, followed by Bonferonni's post hoc test. To compare populations where the means differ greatly from each other, the coefficient of variation was used, which is a unitless and normalized measure of dispersion in a sample, with the equation: $c_v = \frac{\sigma}{\mu}$, where $c_v$ is the coefficient of variation, $\sigma$ is the standard deviation, and $\mu$ is the mean. The mean coefficient of variation across treatments was analyzed using a two-tailed Student’s t-test. $P < 0.05$ was considered significant.
3.4 Results

3.4.1 Experiment 1: Acute TCAP-1 attenuates CRF-induced c-Fos in the limbic system

Data from the saline (n = 9), CRF (n = 9), TCAP (n = 9) and TCAP+CRF (n = 7) groups were analyzed using two-way ANOVA with Bonferroni’s post hoc test, with “TCAP” and “CRF” as factors.

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Figure 3.1: Representative images of 25 µm coronal rat brain sections. Photomicrographs show c-Fos immunoreactivity in the subfields of the hippocampus from the acute TCAP-1 administration study: the CA1 (A-D), CA2 (E-H), CA3 (I-L) and the dentate gyrus (DG; M-P). Rats were cannulated into the lateral ventricle and treated with either saline only (SAL; A, E, I, and M), TCAP-1 and saline (TCAP; B, F, J, and N), saline and CRF (CRF; C, G, K, and O), or TCAP-1 and CRF (TCAP+CRF; D, H, L, and P). Scale bar = 100 µm.
Saline, as expected, did not induce c-Fos protein, which served as a negative control. CRF elicited high c-Fos immunoreactivity in areas associated with the stress response, including the hippocampus (Figure 3.1), amygdala, medial prefrontal cortex (Figure 3.2), dorsal raphe nuclei, and the PVN (not shown).

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Figure 3.2: Representative images of 25 µm coronal rat brain sections. Photomicrographs show the central nucleus of the amygdala (CeA; A-D), the basolateral nucleus of the amygdala (BLA; E-H), and the medial prefrontal cortex (mPFC; I-L) from the acute TCAP-1 administration study. Rats were cannulated into the lateral ventricle and treated with either saline only (SAL; A, E, and I), TCAP-1 and saline (TCAP; B, F, and J), saline and CRF (CRF; C, G, and K), or TCAP-1 and CRF (TCAP+CRF; D, H, and L). Scale bar = 100 µm.

In the hippocampus, the two-way ANOVA revealed that there was a significant main effect of CRF on c-Fos expression in the CA1 (Figure 3.3A; $F_{1,30} = 17.03, p = 0.0003$), CA2 (Figure 3.3B; $F_{1,30} = 19.82, p = 0.0001$), and CA3 (Figure 3.3C; $F_{1,30} = 25.00, p < 0.0001$) subfields, as well as in the dentate gyrus (Figure 3.3D; $F_{1,29} = 33.52, p < 0.0001$). There was a significant main effect of TCAP-1 on c-Fos expression in the CA1 ($F_{1,30} = 4.655, p = 0.0391$) and CA3 ($F_{1,30} = 9.943, p = 0.0037$) subfields as well as in the dentate gyrus ($F_{1,29} = 5.671, p = 0.024$). As well, there was a significant interaction between CRF and TCAP-1 in the CA1 ($F_{1,30} = 5.834, p = 0.022$), CA2
(F\textsubscript{1,30} = 7.276, p = 0.0114), and CA3 (F\textsubscript{1,30} = 13.77, p = 0.0008) subfields of the hippocampus, as well as in the dentate gyrus (F\textsubscript{1,29} = 8.32, p = 0.0073). Bonferroni’s post hoc test revealed that CRF significantly increased c-Fos expression relative to saline in the CA1, CA2, and CA3 subfields of the hippocampus and the dentate gyrus (p < 0.001 for all). Treatment with TCAP-1 in CRF-treated animals significantly reduced c-Fos expression relative to CRF-only treated animals in the CA1 (p < 0.05), CA2 (p < 0.05), and CA3 (p < 0.001) subfields, and the dentate gyrus (p < 0.01).

Figure 3.3: Cumulative c-Fos immunoreactivity counts in the subfields of the hippocampus in the acute TCAP-1 administration study: (A) the CA1, (B) CA2, (C) CA3, and (D) dentate gyrus (DG). CRF significantly increased c-Fos and TCAP-1 significantly blocked the effect of CRF on c-Fos induction across all four subfields of the hippocampus. Values represent means + SEM (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 3.4: Cumulative c-Fos immunoreactivity counts in the subnuclei of the amygdala in the acute TCAP-1 administration study: (A) the central nucleus (CeA), (B) the basolateral nucleus (BLA), and the (C) medial nucleus (MeA). There were significant main effects of CRF in all three nuclei, but TCAP-1 attenuated CRF-induced c-Fos in the CeA and BLA. Values represent means + SEM (*p < 0.05, **p < 0.01, ***p < 0.001).

In the amygdala, two-way ANOVA revealed that CRF had a significant main effect of increasing c-Fos expression in the central nucleus (CeA, Figure 3.4A; $F_{1,30} = 13.89$, $p = 0.0008$) and basolateral nucleus (BLA, Figure 3.4B; $F_{1,30} = 17.16$, $p = 0.0003$). Bonferroni’s post hoc test revealed that treatment of CRF significantly increased c-Fos expression relative to saline in the CeA ($p < 0.05$) and BLA ($p < 0.001$). In these two nuclei, TCAP-1 treatment in CRF-treated animals decreased c-Fos expression such that it was no longer significantly different from saline. CRF had a significant main effect on the medial nucleus of the amygdala (MeA, Figure 3.4C; $F_{1,30} = 7.663$, $p = 0.0096$), but TCAP-1 did not attenuate CRF-induced c-Fos, nor was there any interaction between CRF and TCAP-1.

CRF had a significant main effect of increasing c-Fos immunoreactivity in the medial prefrontal cortex (mPFC, Figure 3.5A; $F_{1,30} = 4.989$, $p = 0.0331$). There was also a main interaction effect between TCAP-1 and CRF ($F_{1,30} = 5.121$, $p = 0.031$). Bonferroni’s post hoc test revealed that CRF significantly increased c-Fos expression relative to saline ($p < 0.05$), and TCAP-1 administration to CRF-treated animals attenuated c-Fos expression so that it did not significantly differ from saline.
Figure 3.5: Cumulative c-Fos immunoreactivity counts in other areas of the brain in the acute TCAP-1 administration study: (A) the medial prefrontal cortex (mPFC), (B) lateral septum, (C) medial septum, and (D) dorsal raphe. There were significant main effects of CRF in all four areas, and TCAP-1 attenuated CRF-induced c-Fos in the mPFC, and septum. TCAP-1 significantly blocked CRF-induced c-Fos in the dorsal raphe. Values represent means + SEM (*p < 0.05, **p < 0.01, ***p < 0.001).

Likewise, CRF had a significant main effect of increasing c-Fos expression in the lateral septum (Figure 3.5B; $F_{1,30} = 16.52$, $p = 0.0003$) and medial septum (Figure 3.5C; $F_{1,30} = 16.44$, $p = 0.0003$). Bonferroni’s post hoc test indicated that CRF significantly increased c-Fos expression relative to saline in the medial and lateral septum ($p < 0.01$ for both). This increase was attenuated by TCAP-1 administration, as TCAP-1 and CRF co-treated animals did not significantly differ from saline controls.
In the dorsal raphe nucleus, CRF had a significant main effect of increasing c-Fos immunoreactivity (Figure 3.5D; $F_{1,30} = 20.45$, $p < 0.0001$), and there was a main interaction effect between CRF and TCAP-1 ($F_{1,30} = 8.32$, $p = 0.0073$). Bonferroni’s post hoc test revealed that CRF significantly increased c-Fos expression relative to saline ($p < 0.001$), whereas TCAP-1 treatment to CRF-treated animals significantly decreased c-Fos levels relative to CRF-only treated animals ($p < 0.05$).

Figure 3.6: Cumulative c-Fos immunoreactivity counts in areas of the brain where TCAP-1 had no effect on c-Fos induction in the acute TCAP-1 administration study: (A) the bed nucleus of the stria terminalis (BnST), (B) parvocellular paraventricular nucleus of the hypothalamus (parvocellular PVN), (C) magnocellular PVN, and (D) locus coeruleus. There were significant main effects of CRF in all four areas; however, TCAP-1 had no effect on c-Fos induction when TCAP-1 and CRF were co-injected together. Values represent means + SEM (*$p < 0.05$, **$p < 0.01$).
CRF significantly increased c-Fos expression in areas associated with the stress response, but not all these areas were sensitive to TCAP-1. CRF had significant main effects in the bed nucleus of the stria terminalis (BnST) (Figure 3.6A; F_{1,30} = 10.28, p = 0.0032), the parvocellular part of the paraventricular nucleus of the hypothalamus (pPVN, Figure 3.6B; F_{1,30} = 15.47, p = 0.0005), magnocellular part of the PVN (mPVN, Figure 3.6C; F_{1,30} = 6.258, p = 0.0180), and the locus coeruleus (Figure 3.6D; F_{1,30} = 9.628, p = 0.0042). Bonferroni post hoc analysis revealed that CRF increased c-Fos expression relative to saline in the PVN (p < 0.05). However, TCAP-1 did not attenuate CRF-induced c-Fos, nor were there any interactions between CRF and TCAP-1 in any of these areas.

### 3.4.2 Experiment 2: Repeated TCAP-1 pre-treatment does not affect CRF-induced c-Fos accumulation

Data from the SAL+SAL (n = 9), SAL+CRF (n = 7), TCAP+SAL (n = 8) and TCAP+CRF (n = 8) groups were analyzed using two-way ANOVA with Bonferroni’s post hoc test, with “TCAP” and “CRF” as factors.

Similar to Experiment 1, saline did not strongly induce c-Fos in any of the areas measured, and served as the negative control. CRF induced c-Fos throughout the brain and served as the positive control.

In the hippocampus, there was a significant main effect of CRF in the CA1 (Figure 3.7A; F_{1,29} = 18.18, p = 0.0002), CA2 (Figure 3.7B; F_{1,29} = 8.631, p = 0.0064), CA3 (Figure 3.7C; F_{1,29} = 22.49, p < 0.0001), and the dentate gyrus (Figure 3.7D; F_{1,29} = 13.42, p = 0.001). However, TCAP-1 had no significant main effects in the CA1 (p = 0.485), CA2 (p = 0.513), CA3 (p = 0.713), or dentate gyrus (p = 0.545), and there were no interactions between TCAP-1 pre-treatment and CRF. Bonferroni’s post hoc test revealed that in the CA1, CA2, and dentate gyrus, there were significant increases in c-Fos immunoreactivity between TCAP+SAL and TCAP+CRF (p < 0.01 for all). In the CA3, there were significant increases in c-Fos immunoreactivity between saline and CRF (p < 0.01) and TCAP+SAL and TCAP+CRF (p < 0.01).
Figure 3.7: Cumulative c-Fos immunoreactivity counts in the subfields of the hippocampus in the repeated TCAP-1 administration study: (A) the CA1, (B) CA2, (C) CA3, and (D) dentate gyrus (DG). There were significant main effects of CRF in all four areas, but TCAP-1 had no effect on CRF-induced c-Fos immunoreactivity. Values represent means + SEM (*p < 0.05, **p < 0.01).
Figure 3.8: Cumulative c-Fos immunoreactivity counts in the subnuclei of the amygdala in the repeated TCAP-1 administration study: (A) the central nucleus (CeA), (B) the basolateral nucleus (BLA), and the (C) medial nucleus (MeA). There were significant main effects of CRF in all three areas, but TCAP-1 had no effect on CRF-induced c-Fos immunoreactivity. Values represent means + SEM (**p < 0.01).

As expected, CRF increased c-Fos levels in the amygdala. There were significant main effects of CRF in the CeA (Figure 3.8A; $F_{1,29} = 14.78$, $p = 0.0006$), BLA (Figure 3.8B; $F_{1,29} = 7.083$, $p = 0.126$), and MeA (Figure 3.8C; $F_{1,29} = 5.587$, $p = 0.025$). There were no main effects of TCAP-1 on the CeA ($p = 0.537$), BLA ($p = 0.902$) or MeA ($p = 0.396$), nor were there any interactions between TCAP pre-treatment and CRF. Bonferroni’s post hoc test indicated that c-Fos induction in the CeA under TCAP+CRF treatment was significantly greater than TCAP-1 ($p < 0.01$).

In the mPFC, CRF had a significant main effect on c-Fos induction (Table 3.1; $F_{1,29} = 25.27$, $p < 0.0001$) but there was no effect of TCAP ($p = 0.640$), nor was there any interaction of TCAP-1 and CRF. Post hoc tests reveal that the CRF group had significantly higher c-Fos levels than saline ($p < 0.01$), and the TCAP+CRF group had significantly higher c-Fos levels than the TCAP group ($p < 0.01$).

CRF had significant main effect in the lateral septum (Table 3.1; $F_{1,29} = 16.01$, $p = 0.0004$) and the medial septum (Table 3.1; $F_{1,29} = 15.39$, $p = 0.0005$). There was no effect of TCAP in either the lateral ($p = 0.789$) or medial ($p = 0.852$), nor were there any interactions between TCAP-1 treatment and CRF. Post hoc analysis revealed that CRF groups had significantly higher c-Fos than saline groups in both the lateral and medial septum ($p < 0.05$ for both) and TCAP+CRF groups had significantly higher c-Fos than TCAP-1 groups in both the lateral and medial septum ($p < 0.05$ for both).
Table 3.1: Cumulative c-Fos immunoreactivity counts in other areas of the brain in the repeated TCAP-1 administration study.

<table>
<thead>
<tr>
<th>Area</th>
<th>Saline+Saline</th>
<th>TCAP+Saline</th>
<th>Saline+CRF</th>
<th>TCAP+CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPFC</td>
<td>1180.4 ± 127.8</td>
<td>1200.6 ± 174.0</td>
<td>2457.4 ± 261.9</td>
<td>2698.6 ± 412.9</td>
</tr>
<tr>
<td>PVN Parvo</td>
<td>166.4 ± 20.5</td>
<td>157.6 ± 18.8</td>
<td>299.9 ± 52.2</td>
<td>296.2 ± 53.2</td>
</tr>
<tr>
<td>PVN Magno</td>
<td>22.9 ± 3.8</td>
<td>22.0 ± 3.6</td>
<td>36.3 ± 4.8</td>
<td>42.2 ± 7.4</td>
</tr>
<tr>
<td>Dorsal Raphe</td>
<td>68.1 ± 8.5</td>
<td>78.9 ± 11.4</td>
<td>145.1 ± 12.9</td>
<td>153.8 ± 26.1</td>
</tr>
<tr>
<td>Locus Coeruleus</td>
<td>37.1 ± 5.9</td>
<td>42.1 ± 8.5</td>
<td>73.9 ± 15.3</td>
<td>83.9 ± 18.5</td>
</tr>
<tr>
<td>Lateral Septum</td>
<td>437.8 ± 53.8</td>
<td>498.4 ± 82.9</td>
<td>939.0 ± 183.2</td>
<td>942.1 ± 134.3</td>
</tr>
<tr>
<td>Medial Septum</td>
<td>111.8 ± 12.8</td>
<td>106.4 ± 17.8</td>
<td>190.3 ± 27.5</td>
<td>188.0 ± 22.8</td>
</tr>
<tr>
<td>BnST</td>
<td>401.9 ± 38.0</td>
<td>388.0 ± 31.8</td>
<td>502.0 ± 48.2</td>
<td>494.3 ± 50.9</td>
</tr>
<tr>
<td>Piriform Cortex</td>
<td>921.6 ± 111.7</td>
<td>1172.1 ± 345.6</td>
<td>1980.6 ± 316.9</td>
<td>2231.3 ± 288.9</td>
</tr>
<tr>
<td>Olfactory Tubercle</td>
<td>133.8 ± 14.5</td>
<td>155.9 ± 34.5</td>
<td>244.3 ± 42.7</td>
<td>211.2 ± 26.7</td>
</tr>
<tr>
<td>CxA</td>
<td>103.0 ± 11.2</td>
<td>115.0 ± 23.9</td>
<td>303.4 ± 68.1</td>
<td>360.3 ± 93.3</td>
</tr>
</tbody>
</table>

BnST, bed nucleus of the stria terminalis; CxA, cortical-amygdala transition area; mPFC, medial prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus.

Likewise, CRF had main effects in the dorsal raphe nucleus (Table 3.1; F₁,29 = 19.90, p = 0.0001), locus coeruleus (Table 3.1; F₁,29 = 8.415, p = 0.007), and BnST (Table 3.1; F₁,29 = 10.28, p = 0.003), but there were no main effects of TCAP-1 in the dorsal raphe (p = 0.573), locus coeruleus (p = 0.583), or BnST (p = 0.816) or any interactions between TCAP-1 and CRF. Post hoc analysis indicated that there c-Fos was significantly higher in CRF groups relative to saline (p < 0.01) and in TCAP+CRF groups relative to TCAP (p < 0.01).

In the olfactory areas, CRF had significant main effects in the piriform cortex (Table 3.1; F₁,29 = 14.91, p = 0.0006), olfactory tubercle (Table 3.1; F₁,29 = 7.750, p = 0.009), and the cortex-amygdalar transition zone (CxA, Table 3.1; F₁,29 = 13.64, p = 0.0009), although there were no significant main effects of TCAP in any of these areas (p = 0.368, p = 0.855, and p = 0.573, respectively). Post hoc analysis revealed that there was significantly higher c-Fos in CRF groups over saline groups in the piriform cortex and olfactory tubercle (p < 0.05 for both) and significantly higher c-Fos in TCAP+CRF groups over TCAP-1 groups in the piriform cortex and CxA (p < 0.05 for both).

Finally, in the PVN, CRF had significant main effects in the pPVN (Table 3.1; F₁,29 = 11.90, p = 0.0017) and the mPVN (Table 3.1; F₁,29 = 10.00, p = 0.0036), but there was no main effect of TCAP (p = 0.876 and p = 0.638, respectively) or interaction between TCAP-1 and CRF. There was significantly more c-Fos induction in TCAP+CRF groups relative to TCAP-1 groups in both the pPVN and mPVN (p < 0.05 for both).
3.4.3 TCAP-1 treatment increases variability in CRF-treated populations

The coefficient of variation ($c_v$) was calculated in groups from Experiment 1 and Experiment 2 to elucidate whether TCAP treatment increases variability in basal and CRF-challenged populations. For each brain area, the $c_v$ was calculated from the group mean and the group standard deviation. The $c_v$ from all brain areas were then averaged for a treatment score to determine the average variation elicited by the treatment.

In experiment 1, there was no difference in the average $c_v$ between TCAP and saline. However, the average $c_v$ was significantly higher in the TCAP+CRF group over the CRF group (Figure 3.9A; $p < 0.05$). Similarly, in experiment 2, there was no difference in the average $c_v$ between TCAP-1 and saline, although the average $c_v$ was significantly higher in the TCAP+CRF group over the CRF group (Figure 3.9B; $p < 0.01$).

As TCAP-1 effects could be dependent on individual differences, the % difference from mean for each area was calculated and plotted for each rat in the TCAP+CRF groups (Figure 3.10A-B). The graphs show that the rats that had high c-Fos levels in one area tended to have high c-Fos throughout their brain, whereas rats that exhibited below-average c-Fos levels in one area tended to have low c-Fos throughout their brain.
Figure 3.9: Mean coefficient of variation ($c_\nu$) in the (A) acute TCAP-1 administration study and the (B) repeated TCAP-1 administration study. TCAP-1 increased the coefficient of variation in the CRF-challenged group in both the acute and repeated TCAP-1 administration studies. Bars represent means $\pm$ SEM ($**p < 0.01$, $***p < 0.001$).

Figure 3.10: Percent difference from mean for 5 rats in the TCAP+CRF group in the (A) acute TCAP-1 administration study and (B) repeated TCAP-1 administration study. Both graphs indicate that in general, rats that had high c-Fos immunoreactivity in one area tended to have high c-Fos immunoreactivity in other areas, whereas rats that had low c-Fos immunoreactivity in one area tended to have low c-Fos immunoreactivity in other areas. Brain areas: (1) CA1; (2) CA2; (3) CA3; (4) DG; (5) MeA; (6) BLA; (7) CeA; (8) mPFC; (9) pPVN; (10) mPVN; (11) lateral septum; (12) medial septum; (13) dorsal raphe; (14) LC; (15) lateral BnST; (16) medial BnST; (17) oval nucleus (BnST).
3.5 Discussion

The aim of the studies in this chapter was to delineate the areas of the brain that are responsive to i.c.v. TCAP-1 administered either acutely or repeatedly, as well as to determine if TCAP-1 can alter CRF-induced activation in the brain. The major finding of these studies is that acute i.c.v. TCAP-1 administration can attenuate CRF-induced c-Fos expression in the hippocampus, central (CeA) and basolateral (BLA) nuclei of the amygdala, medial prefrontal cortex (mPFC), lateral and medial septum, and dorsal raphe nucleus in the male rat brain. Conversely, repeated pre-treatment of TCAP-1 did not attenuate CRF-induced c-Fos in any areas of the brain found in the acute study; however, like the acute study, TCAP-1 treatment increased the coefficient of variation in CRF-treated groups. These studies were the first to map the neuroanatomical activity pattern of TCAP-1, both under basal and stressed conditions, revealing important sites in the limbic system. These findings are consistent with previous in situ hybridization data, which indicated that the limbic system and cortex are structures that strongly express TCAP mRNA (Wang et al., 2005). Thus, these studies provide a strong set of evidence indicating the regions of the brain where TCAP-1 interacts with CRF-associated functions.

The data obtained from the acute TCAP administration study strongly indicated that the hippocampus is a major area in which TCAP-1 exerts its effects. The hippocampus also has a major role in memory and spatial reasoning (Eichenbaum, 2000; Nadel, 1991; Squire and Cave, 1991), and is important in encoding contextual information associated with stressful or fearful environments. The hippocampus is sensitive to stress, and plays a mostly inhibitory role on the HPA axis, although this role is only a small subset of the structure’s functions (Herman et al., 2003). The hippocampus appears to be the most sensitive area to acute TCAP-1 administration in all the brain structures investigated; acute TCAP-1 significantly blocked CRF-induced c-Fos as well as had a significant main effect on c-Fos induction, whereas repeated pre-treatment of TCAP-1 had no effect on CRF-induced c-Fos. Subsequent work by Dhan Chand in our laboratory using a TCAP-1-specific antibody has shown that TCAP-1 is strongly expressed in the CA1, CA2, and CA3 regions of the hippocampus, with weaker staining found in the dentate gyrus (Chand et al., manuscript in preparation). Other subsequent studies have shown that immortalized E14 hippocampal and primary hippocampal culture neurons can take up a fluorescein isothiocyanate (FITC)-[K8]-TCAP-1 analogue in a punctate-like manner, and in some cases it can be translocated to the nucleus where it may associate with chromatin (Chand et al.,
manuscript in preparation). Additionally, as will be discussed in Chapter 4, TCAP-1 can influence cytoskeletal proteins *in vitro* and modulate hippocampal morphology *in vivo*.

The mPFC is associated with the integration and consolidation of stimuli as well as higher-order processes such as decision making and reward. The mPFC is highly interconnected with the limbic system and like the hippocampus, the mPFC selectively inhibits the HPA axis (Herman et al., 2003). The mPFC is differentially activated depending on the type of stress, as mPFC lesions increased PVN c-Fos mRNA in response to restraint stress but not ether (Figueiredo et al., 2003).

In particular, it has been postulated that this brain area is involved in psychological or “anticipatory” stressors: stressors that do not pose a direct threat to the organism, such as footshock (Imaki et al., 1993) or restraint (Cullinan et al., 1995), as opposed to stressors that are physical or “reactive” stressors such as lipopolysaccharide injection (Yokoyama and Sasaki, 1999). I.c.v. CRF induces c-Fos in the mPFC (Bittencourt and Sawchenko, 2000), similar to results in this study. Acute TCAP-1 attenuated CRF-induced c-Fos protein, such that it was no longer significantly different from saline, whereas repeated pre-treatment of TCAP-1 had no effect on CRF-induced c-Fos. Recent work has shown that the mPFC is immunoreactive for TCAP-1 (Chand et al., manuscript in preparation), and therefore, the attenuation of stress-induced processes in the mPFC may impact HPA axis responsiveness.

TCAP-1 administration attenuated CRF-induced c-Fos in the lateral and medial septum. The area appears to respond to psychological stressors, such as restraint or noise (Arnold et al., 1992), but less so to physiological stressors, such as immune challenge (Emmert and Herman, 1999), and contributes inhibitory input to the HPA axis (Herman et al., 2003). I.c.v. CRF induces c-Fos in the medial and lateral septum (Arnold et al., 1992; Bittencourt and Sawchenko, 2000), although the medial septum preferentially expresses CRF₁ receptors whereas the lateral septum expresses CRF₂ receptors (Bittencourt and Sawchenko, 2000). However, infusion of α-helical-CRF, a CRF₁ and CRF₂ receptor antagonist, did not block restraint-induced c-Fos in the lateral septum, indicating that activation in this area may be due to other inputs into the septal region (Arnold et al., 1992). In these studies, acute TCAP-1 attenuated CRF-induced c-Fos in both the medial and lateral septum, indicating that TCAP-1 may have role in regulating the effects of CRF₁ and CRF₂ receptor ligands.
The amygdala plays a vital role in the integration of fear and anxiety (Davis, 1992), and unlike the hippocampus and mPFC, the amygdala plays a mostly excitatory role on the HPA axis (Herman et al., 2003). Three of the main nuclei, the CeA, BLA, and MeA, appear to have differing functions in stress regulation; the CeA appears to be activated in response to physical stressors, whereas the MeA appears to be activated in response to psychological stressors (Dayas et al., 1999, 2001). The BLA has intrinsic connections with other nuclei in the amygdala, and may be important activating the CeA, which is relatively devoid of CRF₁ receptors (Koo et al., 2004). Our experiments confirm that acute CRF induces c-Fos in the CeA, BLA, and MeA, which is similar to previous reports (Bittencourt and Sawchenko, 2000; Imaki et al., 1993). In addition, acute, but not repeated i.c.v. TCAP-1 attenuated CRF-induced c-Fos in the BLA and CeA, but not the MeA, which may indicate that TCAP-1 has a stressor-specific role in stress regulation. We have previously shown that the infusions of TCAP-1 into the BLA can modulate behaviour in the acoustic startle test (Wang et al., 2005), indicating that the amygdala is a sensitive substrate for TCAP-1.

The sensitivity of the dorsal raphe nucleus to acute TCAP-1 suggests that the serotonergic system may play a role in TCAP-1 signalling. This nucleus has serotonergic neurons that express high c-Fos activity during stress (Rioja et al., 2006) and has connections to the mPFC, hippocampus, and amygdala (Lowry, 2002; Vertes, 1991) that are mostly excitatory. However, the dorsal raphe also receives input from the mPFC and CeA (Lee et al., 2003; Peyron et al., 1998) and CRFergic inputs to the dorsal raphe nucleus terminate on GABAergic rather than serotonergic cell dendrites (Waselus et al., 2005). Acute TCAP-1 significantly reduced c-Fos elicited by CRF challenge, and the significant decrease in c-Fos could be a result of direct effects on raphe neurons or reduced input from forebrain afferents. Likewise, reduced c-Fos in forebrain areas such as the limbic system may be a result of reduced serotonergic or GABAergic input.

Several regions of the brain that play a significant role in CRF signalling were not affected by TCAP-1 administration. TCAP-1 did not attenuate CRF-induced c-Fos in either the parvocellular or magnocellular region of the paraventricular nucleus of the hypothalamus (pPVN and mPVN). In particular, the pPVN is the source of CRF to the HPA axis, which indicates that TCAP-1 may act independently of the canonical HPA pathway. These results agree with a previous experiment in that intravenously-administered TCAP-1 did not affect CRF-induced
increases corticosterone secretion (Al Chawaf et al., 2007b). TCAP-1 did not affect the bed nucleus of the stria terminalis (BnST), which is a source of many limbic relays to the PVN (Herman et al., 2005). Likewise, the locus coerulceus, a source of ascending and behaviourally activating noradrenergic input into the forebrain, did not show any TCAP-1 effects. Although experiments have shown that the olfactory areas are immunoreactive for TCAP-1 (Chand et al., manuscript in preparation), there were no effects of TCAP-1 seen in any of these areas.

As a whole, acute TCAP-1 had CRF-attenuating effects in many, but not all forebrain areas measured, indicating a specificity of action instead of a global decrease in activity. TCAP-1 on its own did not significantly induce c-Fos when given acutely nor did it affect basal c-Fos when given as a repeated pre-treatment. This may represent a “primed” state: one where TCAP-1 treatment does not itself induce a response, but instead selectively inhibits the activation of behaviourally relevant brain areas. For example, CRF receptor antagonists, such as CP-154,526 and CRA1000/CRA1001 are inactive in the light/dark box in the absence of stress; however, pre-treatment with a stressor (swim stress) was required to see behavioural effects (Okuyama et al., 1999).

At the completion of these studies, the mechanism by which TCAP-1 attenuates heightened c-Fos remained unknown. Subsequent work by Tanya Nock (Nock, 2009) used reporter assays to dissect apart the promoter sequences of the c-Fos gene to determine which promoter elements were being affected by TCAP-1. The c-Fos gene contains multiple response elements, such as the serum response element (SRE), the activator protein-1 (AP-1) element, and the cAMP response element (CRE). TCAP-1 was previously shown to modulate cAMP accumulation in vitro, such that high TCAP-1 decreased cAMP and low TCAP-1 increased cAMP (Wang et al., 2005). In the signal transduction pathway, cAMP is upstream of the cAMP response element binding protein (CREB) which binds with the AP-1 and CRE. Immortalized N3 hypothalamic cells were transfected with luciferase plasmids containing the promoter sequences, and then treated with TCAP-1. TCAP-1 tended to decrease AP-1 activity and increased basal SRE activity (Nock, 2009). However, there was no effect of TCAP-1 on the basal activity of the c-Fos promoter that contains the CRE, AP-1 and SRE elements. This indicated that there may be additional elements required for c-Fos regulation. The downstream response element (DRE) is downstream of the TATA box and is activated by calcium, which inactivates the DRE-antagonist modulator (DREAM) protein to allow transcription (Carrión et al., 1999). DRE was significantly
decreased following TCAP-1 treatment in transfected N38 cells (Nock, 2009), indicating that the modulation of response elements in concert may be required for a reduction in basal c-fos activity.

In the current study, the best results were seen after acute administration of TCAP-1 under CRF challenge. Although many of the brain areas highlighted in the c-Fos study are rich in CRF₁ expression, Nock (2009) showed that TCAP-1 does not attenuate c-Fos via the CRF receptor. In an elegant study, she transfected 293T cells, which do not bind TCAP-1, with either the CRF₁ or CRF₂ receptor. The transfected 293T cells were treated with CRF and/or TCAP-1 to determine the reporter activity of the CRE, a response element on the c-fos gene. Whereas CRF significantly activated the CRE, indicating CRF receptor binding and c-Fos activation, TCAP-1 administration did not activate the CRE nor did it attenuate CRF-induced CRE activation (Nock, 2009). Furthermore, TCAP-1 did not affect the glucocorticoid response element (GRE) or attenuate dexamethasone induction of the GRE on the crf promoter.

There is also a possibility that the acute mechanisms involved in attenuating CRF-induced c-Fos may differ from long-term mechanisms. Pharmacokinetic data obtained in our laboratory has shown that TCAP-1 has a short half-life in serum when given i.v. (between 3.7-15.8 min) and that TCAP-1 is quickly cleared from the bloodstream, returning to undetectable levels a few hours after bolus injection (Song, Barsyte, and Lovejoy, manuscript in preparation). This suggests that i.c.v. TCAP-1 is cleared in a similarly expedient fashion, such that an acute injection can affect c-Fos when measured two hours later, but repeated TCAP-1 administration is cleared from the brain within hours and changes to c-Fos expression could be absent when measured one week later. However, as TCAP-1 does have long-term behavioural effects (Chapter 2), TCAP-1 may be modifying expression of downstream elements of the CRF response, for example, modulating expression of the CRF receptors to increase the response to stress.

Acute or repeated TCAP-1 treatment in CRF-challenged groups resulted in increased variability relative to CRF-challenged groups, indicating that individual differences may be an important factor. In fact, analysis of the individuals in both acute and repeated TCAP-1 administration groups indicate that the effects of TCAP-1 on CRF-induced c-Fos were consistent across brain areas: some rats had consistently high levels of c-Fos over all their brain areas, whereas some
rats had consistently low levels of c-Fos over all their brain areas. The effectiveness of TCAP-1 in behavioural tests may also rely on individual differences; TCAP-1 administration decreased anxiety-like behaviour in “high emotionality” rats and increased anxiety-like behaviour in “low emotionality” rats (Wang et al., 2005), indicating that the individual differences in responsiveness may help determine the activational/behavioural responses to the peptide.

The current study also provides an indicator of how TCAP-1 may be affecting CRF-induced behaviour (Tan et al., 2008), as TCAP-1 pre-treatment potentiated CRF-induced behaviour in the EPM and open field, whereas it attenuated CRF-induced behaviour in the acoustic startle test. The results may be explained by the role of brain areas required for each task. The amygdala is important in fear and anxiety behaviours, which would be manifested in approach-avoidance conflict tests such as the EPM and open field, as well as in reflexive fear tests such as the acoustic startle test. However, the hippocampus exerts inhibitory control on behavioural responses and may play a larger role in exploration tasks in novel environments such as the EPM and the open field. According the theories by O’Keefe and Nadel (as reviewed by Crusio, 2001), the hippocampus is intimately involved with initiating exploratory behaviour in novel environments to build a cognitive map. Therefore, decreased activation in the hippocampus could result in increased anxiety-like behaviour.

Together, the data in this Chapter indicate that acute, but not repeated injections of TCAP-1 can attenuate CRF-induced c-Fos in specific areas of the brain associated with the extrahypothalamic stress circuitry, particularly in the limbic system and the dorsal raphe nucleus. TCAP-1 did not alter CRF-induced c-Fos in all areas, such as the PVN, BnST, or locus coeruleus, which indicates that TCAP-1 may be acting independently of the HPA axis. Analysis of the coefficient of variation of both acute and repeated treatments indicate that individual differences may play a role in the response to TCAP-1 under stress, as there was no difference in the coefficient of variation between the control groups treated with either saline or TCAP-1; however, TCAP-1 significantly increased the coefficient of variation in groups treated with CRF, such that pre-treatment of TCAP-1, whether acute or repeated, increased the coefficient of variation within the population. Thus, one possibility is that TCAP-1, acting on a receptor system distinct from the CRF receptors, modulates either signal transduction or transcriptional elements downstream of CRF receptor activation.
In light of previous studies indicating that TCAP-1 can also modulate elements of the cytoskeleton (Al Chawaf et al., 2007), the effects that TCAP-1 have on the cell may be more global and could also play a role in neuronal plasticity. If this is so, changes in synaptic interaction or plasticity among cells in CRF-responsive nuclei may modulate the actions of various efferent systems that also modulate the action of CRF.

3.6 References


4 Chapter Four: TCAP-1 and the regulation of neural morphology

Part of this chapter has been published in:


4.1 Abstract

Teneurin C-terminal associated peptide (TCAP)-1 is highly expressed in the hippocampus and amygdala, two areas that are important in regulating behavioural responses to stress. *In vitro*, TCAP-1 upregulates cytoskeletal proteins in immortalized neurons and increases neurite outgrowth in primary hippocampal culture. *In vivo*, TCAP-1 blocks stress-induced c-Fos in the hippocampus and amygdala, and decreases anxiety-like behaviour in the elevated plus maze. This suggests that TCAP-1 plays a role in the remodelling of limbic system networks to modulate stress-induced behaviours. Different chronic stressors, such as social defeat or restraint, cause a remodelling of the pyramidal neurons in the hippocampus and amygdala. Both areas are neuroplastic, and dendritic spines are sensitive to stress and receive excitatory synaptic connections. In this study, repeated daily injection of TCAP-1 (300 pmol) for 10 days increased spine density in the CA1 and CA3 regions of the hippocampus, without affecting spine density in the amygdala. Interestingly, 10 days of restraint increased spine density in CA1 and CA3 neurons. Repeated TCAP-1 had no effect on spine density under restraint conditions; however, linear regressions indicate that there may be a correlation between spine density and EPM behaviour, a relationship that changes when treated with TCAP-1.

4.2 Introduction

Teneurin C-terminal associated peptide (TCAP)-1 is a novel neuropeptide found in the metazoan central nervous system. *In situ* hybridization experiments indicate that TCAP-1 is expressed in the limbic system, especially in the pyramidal layer of the hippocampus and in neurons of the basolateral nucleus of the amygdala (BLA) (Wang et al., 2005). TCAP-1 is neuroprotective against alkalotic stress (Trubiani et al., 2007), and influences brain-derived neurotrophic factor (BDNF) expression and translation (Ng, 2010) in immortalized hypothalamic neurons.
Moreover, acutely-administered TCAP-1 attenuates CRF-induced c-Fos accumulation in the limbic system, notably in the hippocampus and amygdala (Chapter 3; Tan et al., 2009) by attenuation of the AP-1 and DRE response elements on the c-fos gene (Nock, 2009). Five days of repeated injections of TCAP-1 in a pre-treatment regimen modulated CRF-induced behaviour whereas ten days of TCAP-1 reduced anxiety-like behaviour under basal conditions (Chapter 2; Tan et al., 2008). Furthermore, acute administration of TCAP-1 into the BLA of rats modulated acoustic startle, and i.c.v. injections of TCAP-1 produced behavioural effects that lasted at least 3 weeks after treatment (Wang et al., 2005), indicating that TCAP-1 has long-lasting effects. Although these studies indicate that TCAP-1 may have effects on CRF regulation, and hence, elements of the stress response, the mechanism by which this occurs is not known.

The hippocampus and amygdala are structures that are associated with the regulation of the behavioural stress response (Chapter 3; Herman et al., 2003, 2005; Jacobson and Sapolsky, 1991) and recent data indicate that these areas are important substrates for TCAP-1 action. Dendrites on the pyramidal neurons of these two regions are morphologically plastic, and dendritic branches can reversibly change in complexity in response to a variety of stimuli, such as stress (Lambert et al., 1998; Magariños et al., 1996; Watanabe et al., 1992) and learning (Mahajan and Desiraju, 1988). The dendrites of these two areas contain numerous spines that form some of the incoming excitatory synapses. The formation and pruning of spines is also highly dynamic and are more sensitive to stimuli than the remodelling of dendritic trees (Dalla et al., 2009; Morales-Medina et al., 2009; Shors et al., 2001; Silva- Gómez et al., 2003). In particular, chronic restraint or immobilization drastically alters the number of dendritic spines in the CA1 and CA3 regions of the hippocampus (McLaughlin et al., 2005; Sunanda et al., 1995) and the BLA (Mitra et al., 2005; Vyas et al., 2006), which involves remodelling of cytoskeletal proteins and other elements (Gu et al., 2008; Tada and Sheng, 2006). In vitro, TCAP-1 increases cytoskeletal proteins, including β-actin and β-tubulin, and modulates neurite outgrowth in immortalized hypothalamic neurons, and increases neurite outgrowth in primary hippocampal neurons (Al Chawaf et al., 2007a).

Inputs into the dendrites of the hippocampus are laminar (Figure 4.1), such that they receive input from sources unique to each stratum (Amaral and Lavenex, 2007; Knowles, 1992). Cortical input arrives from the entorhinal cortex, which sends projections to the dentate gyrus via the perforant path, as well as directly to the distal apical dendrites of CA1 and CA3 neurons, a
layer known as the stratum lacunosum-moleculare. Dentate gyrus mossy fibre input extends to the thorny excrescences of the stratum lucidum, a layer proximal to the soma, of CA3 neurons. CA3 axons project to the CA1 via the Schaffer collaterals to the stratum radiatum, a layer in between the stratum lacunosum-moleculare and lucidum, and to the stratum oriens, which exists on the basilar tree. The CA1 then projects to the subiculum, one of the main outputs of the hippocampus. Intrinsically, axons of the CA3 project their axons both contralaterally and ipsilaterally to other CA3 neurons in addition to CA1 neurons, as well as to interneurons within the layer (Amaral and Lavenex, 2007). These projections terminate in the strata radiatum and oriens of CA3 neurons.

**Figure 4.1: Pathways of the hippocampal formation.** (A) In the hippocampus, information is unidirectional and arrives from the entorhinal cortex (ECx) via the perforant path (PP), arriving at the dentate gyrus (DG) mossy fibres. These fibres project to the thorny excrescences of CA3 pyramidal neurons. Direct input from the ECx via the PP projects to the distal dendrites of CA3 neurons. The CA3 sends associational and commissural collaterals to other CA3 neurons both contralaterally and ipsilaterally. The CA3 also sends axons to CA1 neurons via the Schaffer Collateral pathway. CA1 neurons then project to the subiculum (Sb) which projects to the ECx. Inputs to the pyramidal neuron dendrites are laminar (red box, CA3). (B) For example, in CA3 neurons, in the apical tree, the distal most part of the dendrites (stratum lacunosum-moleculare) receives information from the PP. The middle part of the apical tree (stratum radiatum) receives commissural and associational collaterals from other CA3 neurons. The proximal part of the apical tree (stratum lucidum) receives axons from the dentate gyrus mossy fibres. In the basilar tree, the stratum oriens receives commissural intrinsic connections. Scale bar = 100 µm. Adapted from www.bristol.ac.uk/synaptic/pathways.
Previous work has shown that 10 days of TCAP-1 can modulate dendritic morphology in CA3 hippocampal neurons under unstressed and restraint conditions (Al Chawaf, 2008). In unstressed conditions, TCAP-1 treatment decreased dendritic branching in the basilar tree 90-130 µm away from the soma, an area that corresponds to the stratum oriens. Under restraint conditions, TCAP-1 treatment increased dendritic branching in the apical tree 250-300 µm away from the soma, which corresponds to the stratum radiatum. This indicated that TCAP-1 may have a layer-specific role in modulating pyramidal neuron morphology, and dendritic spines were investigated as a more sensitive measure of stress-induced changes in morphological plasticity than dendritic arbour, as some stress-induced spine changes can be seen after a single stress episode (Mitra et al., 2005; Shors et al., 2001). A recent study indicated that a 5 h multimodal bout of stressors resulted in loss of dendritic spines in the CA3 stratum radiatum (Chen et al., 2010), indicating that stimuli can differentially alter strata in the hippocampus. Furthermore, this effect could be blocked by i.c.v. infusion of a CRF1 receptor blocker (NBI 30775), which prevented spine loss and restored memory function.

I hypothesized that as 10 days of TCAP-1 treatment significantly affected dendrite branching in CA3 neurons, TCAP-1 should have a role in remodelling dendritic spines in the CA1 and CA3 of the hippocampus as well. In addition, the BLA was analyzed, as it highly expresses TCAP-1 mRNA and injections to the BLA modulate anxiety-like behaviour. I propose that changes in dendritic spines underlie modifications of anxiety-like behaviour, and that these spine changes may be a mechanism for long-term behavioural effects seen in previous studies.

4.3 Materials and Methods

4.3.1 Animals

All experiments were performed using methods approved by the University of Toronto Animal Care Committee and the Canadian Council on Animal Care. Male Wistar rats (n=30, 250-275 g, Charles River Laboratories, Montreal, QC) were individually housed in shoebox cages on a 12 h light/dark cycle (lights on at 0700 h) at a constant temperature of 21 °C, and were provided with standard rat chow and tap water ad libitum.
4.3.2 Surgery

Upon delivery, rats were allowed one week to acclimatize to housing conditions. Rats were anaesthetized with 2-3% isoflurane (mixed with oxygen) and fit into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Prior to surgery, rats were administered subcutaneous analgesics (0.5 mg/kg buprenorphine). Rats were then surgically implanted with a 22-gauge guide cannula (Plastics One, Roanoke, VA) into the right lateral ventricle (AP: -1.0 and ML: -1.4 from bregma, DV: -3.7 from dura, co-ordinates according to Paxinos and Watson, 1998). The guide cannula was secured to the skull using four jeweller’s screws and dental acrylic, and the opening was covered with a cannula dummy to ensure cannula patency. The wound was treated with Hibitane (Pfizer Animal Health, Kirkland, QC) to prevent infection. Rats were allowed to recover from anaesthesia in clean cages under warm heating lamps. Rats were then transferred to a clean cage and provided with wet chow and Nutri-Cal (Vetoquin USA, Fort Worth, TX) for 12 h. Rats were then transferred to a clean cage and rats were allowed one week to recover from surgery.

4.3.3 TCAP-1 and vehicle administration

Synthetic TCAP-1 was synthesized as previously described (Wang et al., 2005). The lyophilized peptide was solubilized by exposure to ammonium hydroxide vapour for 2 min before dilution in phosphate buffered saline (10 mM PBS, pH 7.4) at a concentration of 10^{-4} M.

Injections were delivered between 1000 h and 1200 h in a randomized order. Intracerebroventricular (i.c.v.) injections were delivered at a rate of 2 µl/min by a syringe pump (Razel Scientific Instrument Inc., Stamford, CT) connected by PE-50 tubing to a 28-gauge stainless steel injector that extended 1 mm below the cannula guide opening. Animals were injected once daily for 10 consecutive days with either 3 µl of saline (control) or 3 µl of 10^{-4} M TCAP-1. The injector needle was left in place for 60 s following injection to allow for the peptide to diffuse into the CSF and to prevent backflow up the cannula.

4.3.4 Restraint protocol

Rats were either handled lightly and run through the restraint tube (for rats to experience the novelty of the tube) as a non-stressed control, or were subjected to 2 h per day for 10 consecutive days of restraint in a clear Plexiglas tube (length = 15.8 cm; interior diameter = 7 cm) in their
home cages. Restraint took place between 1300 h and 1500 h each day after completion of daily i.c.v. injections.

4.3.5 Tissue fixation and histology

Twenty-four hours after the last injection/restraint treatment, rats were deeply anaesthetized with 3% isoflurane in oxygen and decapitated. The brain was removed within 2 min and sectioned to remove the olfactory lobe and 2-3 mm of the anterior part of the frontal lobe and the brainstem and cerebellum to yield a tissue block between AP 1.0 mm and -7.0 mm from bregma (coordinates according to Paxinos and Watson, 1998). Brain blocks were trimmed to allow Golgi stain to fully impregnate the tissue block. Fresh brain blocks were rinsed with PBS and placed into a Golgi impregnation solution containing potassium dichromate, mercuric chloride, and potassium chromate (Solutions A and B, Rapid GolgiStain kit, FD Neurotechnologies Inc., Ellicott City, MD), and were stored in the dark in separate scintillation tubes for 14 days. Brains were then rinsed in PBS and transferred to a cryoprotectant solution (Solution C, Rapid GolgiStain kit) for 48 h, rapidly frozen in -70 °C isopentane and stored at -80 °C. Brain blocks were mounted on the cryostat pedestal using Tissue-Tek OCT media (Sakura Finetek, Torrance, CA). Brains were sectioned on a cryostat (Leica CM3050, Richmond Hill, ON) at -30 °C to obtain 100 µm brain slices on the coronal plane through the amygdala and the dorsal hippocampus. Brain sections were thaw-mounted with a drop of water on 3% gelatinized slides, air-dried in the dark, and stored in slide boxes at room temperature prior to staining. The brain sections were then rehydrated in ddH₂O and incubated in proprietary solution (Solutions D and E) which visualizes neurons previously impregnated with Golgi stain. The sections were then dehydrated in graded ethanols, cleared in xylene, and cover-slipped with Permount and coverglass (Fisher Scientific, Ottawa, ON). Slides were subsequently stored in the dark until analysis. All brains were checked using a light microscope for proper cannula placement into the lateral ventricle, and those with improper placement were removed from the experiment.

4.3.6 Dendritic spine analysis

Pyramidal cells from the CA1 and CA3 regions of the hippocampus (between bregma: -2.30 to 4.80 mm, Paxinos and Watson, 1998) and from the BLA (between bregma: -1.80 to -3.14 mm) were selected for quantitative analysis. For each brain, 5 CA1, 5 CA3, and 5 BLA neurons were analyzed. To be included in the analysis, Golgi-impregnated pyramidal neurons had to be
uniformly stained throughout the basilar and apical trees and discernible from neighbouring cells. Because inputs to the CA1 and CA3 neuron dendrites are laminar, the major strata of each tree were analyzed separately. For the CA1 and CA3 regions, spines on primary and secondary branches in the stratum oriens in the basilar tree, and spines on secondary and tertiary dendritic branches in the stratum radiatum and stratum lacunosum-moleculare of the apical tree were investigated. In the BLA, spines on primary and secondary branches were quantified. For each area, 5 segments of 10 µm each were photographed using an inverted light microscope at 1000x magnification with oil immersion (Leica DMI3000, Richmond Hill, ON). In some cases, the segments were from the same branch. Dendritic spines from photomicrographs were manually counted using NIS Elements Software Basic v2.30 (Nikon, Mississauga, ON) by an observer blind to the rat treatments. Spines were required to be distinct from the dendritic branch to be counted.

4.3.7 Statistical analysis

For purposes of the analysis, rats were divided into non-restraint groups and restraint groups. Spine density counts from each neuron were averaged for a cell mean, and five neurons from each animal were averaged for an animal mean for each of the strata analyzed. To determine the effect of TCAP-1 on spine density under basal (unstressed) conditions, in the CA1 and CA3 areas of the hippocampus, strata were analyzed by two-way ANOVA between the saline + no restraint and the TCAP-1 + no restraint groups, with TCAP-1 treatment and strata as independent factors. For the BLA, individual student’s t-tests were performed. To determine the effects of restraint on spine density, two-way ANOVAs were performed between saline + no restraint and saline + restraint groups, with restraint and strata as independent factors. For the BLA, individual t-tests between the saline + no restraint and saline + restraint groups were performed. Finally, to determine the effect of TCAP-1 on spine density under restraint (stressed) conditions, two-way ANOVAs were performed in the restraint groups with TCAP-1 treatment and strata as independent factors. For the BLA, individual student’s t-tests were performed in the restraint groups. Data obtained from the elevated plus maze that was an adjunct to this study (see Chapter 2) were analyzed in conjunction with spine density; rats that had completed the EPM and had brain areas analyzed for spine density measures were included in a linear regression correlation analysis. Data was analyzed using GraphPad Prism 4.0. P < 0.05 was considered significant.
4.4 Results

In each of the treatment groups, Golgi-stained neurons in the CA1 and CA3 areas of the hippocampus and pyramidal-like neurons of the BLA were easily identifiable. Representative dendritic segments are illustrated in Figure 4.2A and Figure 4.4A (CA1 hippocampus), Figure 4.3A and Figure 4.5A (CA3 hippocampus) and Figure 4.6A (BLA).

4.4.1 Effects of TCAP-1 on spine density under basal conditions

Animal means were analyzed by two-way ANOVA with TCAP-1 and stratum as independent factors. In the CA1 region of the hippocampus (Figure 4.2B), TCAP-1 treatment had a significant main effect on spine density. (n = 5; F1,24 = 8.162, p = 0.0087). There was also a significant main effect of stratum (F2, 24 = 6.151, p = 0.007), indicating that there is a difference in spine density across the different strata. There was no interaction between spine density and stratum (F2,24 = 0.4711, p = 0.63). Bonferronni’s post hoc tests indicate that the stratum radiatum possessed a significantly greater number of spines than the strata lacunosum-moleculare or oriens (p < 0.05).
Figure 4.2: Effects of repeated injections of TCAP-1 on dendritic spine density in the CA1 region of the hippocampus. (A) Representative photomicrographs of Golgi-stained dendrites of CA1 pyramidal neurons show three laminar regions (stratum lacunosum-moleculare [SLM] and stratum radiatum [SR] of the apical tree and stratum oriens [SO] of the basilar tree) treated with saline + no restraint or TCAP-1 + no restraint. (B) Spine density counts from secondary and tertiary branches of dendrites in the SLM and SR regions and counts from primary and secondary branches of the SO region. TCAP-1 had significant main effects on spine density ($F_{1,24} = 8.162, p = 0.0087$). Bars are mean + SEM with $n = 5$ rats per group ($5 \times 10 \, \mu m$ segments measured for 5 neurons per animal). Scale bar = 10 μm.

In the CA3 region of the hippocampus, (Figure 4.3B), TCAP-1 treatment had a significant main effect on spine density ($n = 5; F_{1,24} = 18.26, p = 0.0003$), however, the stratum did not have any significant main effects ($F_{2,24} = 0.0726, p = 0.9302$), nor were there any interactions between TCAP-1 and stratum ($F_{2,24} = 0.0848, p = 0.9190$).

TCAP-1 did not affect spine density in the BLA region ($n = 5; \text{Figure 4.6B}; p = 0.2918, \text{t-test}$).
Figure 4.3: Effects of repeated injections of TCAP-1 on dendritic spine density in the CA3 region of the hippocampus. (A) Representative photomicrographs of Golgi-stained dendrites of CA3 pyramidal neurons show three laminar regions (SLM, SR, and SO) treated with saline + no restraint or TCAP-1 + no restraint. (B) Spine density counts from secondary and tertiary branches of dendrites in the SLM and SR regions and counts from primary and secondary branches of the SO region. TCAP-1 had significant main effects on spine density ($F_{1,24} = 18.26$, $p = 0.0003$). Bars are mean + SEM with $n = 5$ rats per group (5 x 10 μm segments measured for 5 neurons per animal). Scale bar = 10 μm.

4.4.2 Effects of TCAP-1 on spine density under stressed conditions

Analyses were performed between saline-treated animals in non-restrained and restrained groups. Animal means were analyzed by two-way ANOVA with restraint and stratum as independent factors. In the saline treated groups, restraint had significant main effects in the CA1 ($n = 5; F_{1,24} = 5.071$, $p = 0.0338$) and the CA3 ($F_{1,24} = 8.570$, $p = 0.0074$), indicating that restraint, indeed, increased spine density in the hippocampus, and could be a control to investigate how TCAP-1 affects spine density in stressed conditions. However, restraint did not significantly alter spine density in the BLA ($p = 0.3436$, t-test).
Figure 4.4: Effects of repeated injections of TCAP-1 on dendritic spine density in the CA1 region of the hippocampus under repeated restraint conditions. (A) Representative photomicrographs of Golgi-stained dendrites of CA1 pyramidal neurons show three laminar regions (SLM, SR, and SO) treated with saline + restraint or TCAP-1 + restraint. (B) Spine density counts from secondary and tertiary branches of dendrites in the SLM and SR regions and counts from primary and secondary branches of the SO region. TCAP-1 had no significant effects on spine density. Bars are mean + SEM with n = 5 rats per group (5 x 10 μm segments measured for 5 neurons per animal). Scale bar = 10 μm.

Figure 4.5: Effects of repeated injections of TCAP-1 on dendritic spine density in the CA3 region of the hippocampus under repeated restraint conditions. (A) Representative photomicrographs of Golgi-stained dendrites of CA3 pyramidal neurons show three laminar regions (SLM, SR, and SO) treated with saline + restraint or TCAP-1 + restraint. (B) Spine density counts from secondary and tertiary branches of dendrites in the SLM and SR regions and counts from primary and secondary branches of the SO region. TCAP-1 had no significant effects on spine density. Bars are mean + SEM with n = 5 rats per group (5 x 10 μm segments measured for 5 neurons per animal). Scale bar = 10 μm.
Data from restrained animals (animal means) were analyzed by two-way ANOVA with TCAP-1 and stratum as independent factors. In the CA1 region (Figure 4.4B), TCAP-1 did not have any significant main effects (n = 5; F_{1,24} = 1.365, p = 0.2541), although there was a significant main effect of stratum (F_{2,24} = 4.711, p = 0.0188) but no interaction between TCAP-1 and stratum (F_{2,24} = 0.7729, p = 0.4728). In the CA3 region (Figure 4.5B), there were no main effects of TCAP-1 (F_{1,24} = 0.5681, p = 0.4583), stratum (F_{2,24} = 0.04943, p = 0.9519), or interaction of TCAP-1 and stratum (F_{2,24} = 0.7729, p = 0.4728).

There was no effect of TCAP-1 in the BLA (n = 5; Figure 4.6C; p = 0.6212, t-test).

**Figure 4.6: Effects of repeated injections of TCAP-1 on dendritic spine density in the BLA under non-restraint and repeated restraint conditions.** (A) Representative photomicrographs of Golgi-stained dendrites of BLA pyramidal-like neurons treated with saline + no restraint, TCAP-1 + no restraint, saline + restraint, and TCAP-1 + restraint conditions. (B) Spine density counts from primary and secondary branches of dendrites under non-restraint conditions and (C) restraint conditions. TCAP-1 had no significant effects on spine density in the BLA, and restraint did not cause a change in spine density. Bars are mean + SEM with n = 5 rats per group (5 x 10 μm segments measured for 5 neurons per animal). Scale bar = 10 μm.

### 4.4.3 Linear regression analysis

Linear regression analysis was performed on all rats that had their brains analyzed for spine density, and had completed the EPM test (Table 4.1, Figure 4.7). In the CA1 or BLA, no
consistent correlations could be drawn between spine density and behavioural measures on the EPM. However, in the stratum radiatum of the CA3, there was a strong positive correlation between spine density and open arm entries in the maze in rats treated with saline + restraint (n = 4; $r^2 = 0.7975$, $p < 0.0001$), and a negative correlation in the TCAP + restraint group (n = 3; $r^2 = 0.8204$, $p < 0.0001$). Similar results were seen in correlations of the same measures in the stratum lacunosum-moleculare and oriens (data not shown). Negative correlations in the stratum radiatum of the CA3 between spine density and open arm time were also found in TCAP + no restraint (n = 4; $r^2 = 0.3479$, $p = 0.0062$) and TCAP + restraint groups (n = 3; $r^2 = 0.2781$, $p = 0.0434$). Finally, there was a positive correlation between spine density and closed arm entries in the TCAP + no restraint (n = 3; $r^2 = 0.63$, $p < 0.0001$) and a negative correlation between spine density and closed arm entries in the TCAP + restraint group (n = 4; $r^2 = 0.3947$, $p = 0.0121$).

Table 4.1: Raw behaviour of rats in the EPM subjected to 10 days of TCAP-1 injections and restraint.

<table>
<thead>
<tr>
<th></th>
<th>Saline+No Restraint</th>
<th>TCAP+No Restraint</th>
<th>Saline+Restraint</th>
<th>TCAP+Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Arm Entries</td>
<td>8.75 ± 2.4</td>
<td>8.6 ± 1.9</td>
<td>9.6 ± 2.2</td>
<td>8.3 ± 0.8</td>
</tr>
<tr>
<td>Open Arm Time (s)</td>
<td>113.2 ± 13.1</td>
<td>150.0 ± 8.5</td>
<td>147.1 ± 20.0</td>
<td>119.9 ± 10.3</td>
</tr>
<tr>
<td>Closed Arm Entries</td>
<td>8.75 ± 1.1</td>
<td>4.2 ± 0.7</td>
<td>6.2 ± 1.7</td>
<td>7.3 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

Figure 4.7: Correlations between spine density in the stratum radiatum (SR) of the CA3 and EPM behavior. Linear regression analyses indicated correlations between spine density of the CA3 SR and (A) open arm entries, (B) open arm time, and (C) closed arm entries.
4.5 Discussion

TCAP is a novel neuropeptide that is strongly expressed in the hippocampus, amygdala, and other nuclei in the limbic system (Wang et al., 2005). The present studies show that TCAP-1, when injected i.c.v. over a period of 10 days, increases spine density in CA1 and CA3 hippocampal neurons under basal conditions but does not affect BLA neurons. TCAP-1 did not affect spine density in restrained rats in the hippocampus or BLA. Furthermore, performance on the elevated plus maze (EPM, Chapter 2) correlated with spine density changes in CA3 neurons. Thus, these studies indicate that TCAP-1 has the potential to modulate neuronal plasticity and could therefore facilitate the actions of afferent fibres regulating stress-sensitive regions of the brain.

Previous work had demonstrated that 10 days of TCAP-1 administration can cause a remodelling of the dendrites of CA3 hippocampal neurons in areas corresponding to the stratum radiatum and stratum oriens of the dendritic trees (Al Chawaf, 2008). These results indicated that TCAP-1 may be selectively affecting different parts of the dendritic trees. As inputs to the CA3 are laminar, and these two areas receive intrinsic connections both contralaterally and ipsilaterally from within the CA3 (Amaral and Lavenex, 2007), I hypothesized that TCAP-1 may be modulating synaptic connectivity within these layers.

Under non-restrained conditions, TCAP-1 increases spine density in the CA1 and CA3 regions across all the strata of the dendritic trees. There was a significant effect of strata in the CA1 region, as the stratum radiatum had more spines than the strata lacunosum-moleculare or oriens. In the CA3, there was no effect of stratum, nor were there any interactions between spine density and stratum, indicating that whereas TCAP-1 increases spine density in the hippocampus, it does not differentially affect the spines on the different strata of the dendritic trees. TCAP-1 did not affect BLA neurons under basal conditions, although the BLA is a site of TCAP-1 mRNA expression (Wang et al., 2005), immunoreactivity (Chand et al., manuscript in preparation), CRF attenuation (Tan et al., 2009), and behavioural activity (Wang et al., 2005). The observed changes in spine formation provide a mechanism that explains, in part, the changes in the EPM behaviour (Chapter 2). Ten days of injections of TCAP-1 significantly increased open arm time and decreased closed arm entries on the EPM.
The increase in CA3 spine density in the stratum oriens but decrease in basilar branching indicates that overall spine availability may remain relatively unchanged in the basilar tree, as spine density as a whole changes as a function of dendritic length (Radley and Morrison, 2005). However, in the apical tree, there was no change in the branching but an increase in spine density, suggesting an increase in available spines. Together, this indicates that there is an increase in the apical, but not necessarily basal dendritic spine availability of CA3 neurons, although one should note that an increase of spines does not necessarily equate with an increase in synapses, as a proportion of synapses are “silent” and lack AMPA receptors (Bourne and Harris, 2008). Further studies that investigate the role of TCAP-1 on the type of spines and their receptor disposition will be required to determine if TCAP-1 has a role in increasing excitatory synapse input into the CA3 region.

These studies provide new insight into the role of TCAP-1 in the hippocampus and support previous studies indicating a significant role in this region of the brain. The pyramidal neurons of the hippocampal CA3 region are strongly immunoreactive for TCAP-1, whereas the CA1 region is TCAP-1-immunoreactive, albeit less than the CA3 (Chand et al., manuscript in preparation). Recent in vitro experiments have demonstrated that primary hippocampal neurons readily take up fluorescein isothiocyanate (FITC)-labelled TCAP-1, after which it is trafficked to the cytosol, and in some cases, to the nucleus (Chand et al., manuscript in preparation). My observations that TCAP-1 induces changes in spine density indicate that TCAP-1 must have a role in cytoskeletal element regulation. Dendritic spine dynamics depend on the actin filaments in the cytoskeleton, which mediate the formation, elimination, and stability of spines. In addition, the cytoskeleton is also dependent on the regulation of microtubules, where new spines anchor their actin filaments (Hotulainen and Hoogenraad, 2010). Within the neuron, TCAP-1 increases the cytoskeletal proteins, β-actin and β-tubulin, and modulates neurite outgrowth in immortalized hypothalamic culture (Al Chawaf et al., 2007a). Furthermore, in vitro knockdown of all four TCAPs using siRNA caused a significant decrease in neurite number in N38 cells (Trubiani, 2008), indicating that TCAPs may be important for neurite stabilization. Recent data has shown that 100 nM TCAP-1 administration on immortalized hippocampal E14 cells causes a remodelling of the cytoskeleton and reorganization of α-tubulin, β-tubulin, and β-actin to the periphery and neurites of the cell (Dhan Chand, personal communications). Our laboratory has indicated that TCAP-1 binds to several 30-70 kDa proteins, one of which is a 35 kDa binding
protein, identified by mass spectrometry as elongation factor-1 (EF-1; Dhan Chand, personal communications). One isoform of the protein, EF-1α, is expressed in pyramidal cell bodies and dendrites of the hippocampus, and has been localized in the strata radiatum and oriens, which receive associational and commissural afferents (Huang et al., 2005). Interestingly, EF-1α binds actin and may anchor β-actin mRNA to the cytoskeleton in dendrites (Liu et al., 2002). EF-1α is also increased after perforant path activation although this may represent a reorganization of the actin cytoskeleton to redistribute EF-1α to dendritic areas. EF-1 co-localizes with endogenous TCAP-1 in the cytosol of E14 hippocampal cells (Dhan Chand, personal communications); however, FITC-labelled TCAP-1 does not co-localize with EF-1, as exogenous TCAP-1 appears to be sequestered in vesicles. However, it is possible that TCAP-1 treatment can increase expression of endogenous TCAP-1, which would bind to EF-1α to exert cytoskeletal changes.

TCAP-1 also binds to the 15 kDa protein, stathmin (Dhan Chand, personal communications), which is involved in the decoupling of α,β-tubulin heterodimers (Cassimeris, 2002). Stathmin mRNA is increased in the hippocampus following a single but not chronic methamphetamine dose, a treatment which increases dendritic spines (Ujiike et al., 2002). This indicates that stathmin may only be required for spine sprouting rather than upkeep. However, it remains to be seen whether TCAP-1 can affect other components of the actin cytoskeleton, such as microtubule-associated protein-2 (MAP2) and synaptopodin, which have been implicated in anxiety disorders in human patients (Soetanto et al., 2010), and MAP1 and synaptophysin, which are increased and decreased respectively in the hippocampus after restraint (Xu et al., 2004).

Neurotrophins like brain-derived neurotrophic factor (BDNF), may also play a role in the upkeep of hippocampal complexity, as haploinsufficient BDNF+/− mice have simplified hippocampal branching and are resistant to stress-induced atrophy (Magariños et al., 2010). TCAP-1 has been shown to reduce BDNF in vitro (Ng, 2010), indicating that a decrease of neurotrophic support may be responsible for a lack of effect in restraint populations.

Restraint treatment increased spine density in saline-treated controls relative to unstressed controls, similar to some studies (McLaughlin et al., 2005; Sunanda et al., 1995). Some stressors, such as acute tail shock, increased CA1 spines (Shors et al., 2001). However, other stressors, such as prenatal stress (Martínez-Téllez et al., 2009), postweaning social isolation
(Silva-Gómez et al., 2003), corticosterone treatment (Morales-Medina et al., 2009), or oxidative stressor (Avila-Costa et al., 1999) reduce spine density in the hippocampus. Stress-induced spine loss has been attributed to high corticosterone working synergistically with excitatory amino acids (for a review, see Fuchs and Flügge, 1998). However, the increase in spines seen in this study may be a result of habituation to the restraint conditions, as repeated restraint results in decreased ACTH secretion (for a review, see Martí and Armario, 1998) and corticosterone (Girotti et al., 2006; Magariños and McEwen, 1995) over time. Restraint is regarded as a mild emotional stressor and is less severe than immobilization (Briski and Gillen, 2001), a stressor treatment that was used to induce dendritic spine changes over 10 days of stress (Mitra et al., 2005). In this study, mild restraint, instead of inducing a reduction in spines associated with other stressors, could have produced an arousing environment which increased possible synaptic connections, and the predictability of the stressor could have resulted in an increase in spines. For example, Parihar et al. (2011) have shown that predictable chronic mild stress, in contrast to unpredictable mild stress, results in increased open arm time in the elevated plus maze, increased cognitive ability in the Morris water maze, and an increase in neurogenesis in the hippocampus. Alternatively, Sunanda et al. (1995) suggest that the increase in spines could be due to abnormal sprouting of new spines, such as that induced by a brief seizure, enough to induce long-term potentiation (Ben-Ari and Represa, 1990). Unfortunately, blood samples to measure corticosterone levels were not obtained so we could not determine if repeated restraint treatment resulted in levels of corticosterone lower than other stressors (i.e. immobilization, oxidative stressor).

Unlike other experiments where a stressor was required to see effects (Al Chawaf et al., 2007b; Tan et al., 2008, 2009), 10 days of TCAP-1 administration did not appear to have effects on dendritic spines under restraint conditions relative to saline-treated restraint controls in the hippocampus or amygdala. However, an increase in spines may compensate for a loss in surface area due to decreased branching (Radley and Morrison, 2005). Similar to other studies in the literature (Magariños and McEwen, 1995; Vyas et al., 2002), 10 days of restraint reduced branching in the apical shaft of CA3 neurons (both the strata radiatum and lacunosum-moleculare) without affecting the basilar tree (Al Chawaf, 2008 Thesis). TCAP-1 treatment in restrained rats also increased apical branching of CA3 neurons, whereas the basilar tree was unchanged (Al Chawaf, 2008). Therefore, restraint decreased branching but increased spines in
the apical tree of CA3 neurons in saline-treated rats, indicating that overall there may be no change in spine availability. However, TCAP-1 treatment both increased dendritic branching and increased spine density in the apical tree of CA3 neurons, suggesting and increase in spines 250-300 µm away from the soma, an area corresponding to the stratum radiatum. This suggests that TCAP-1 may indeed increase available spines particularly in the layer of the hippocampus that receives intrinsic input from other ipsilateral and contralateral CA3 neurons. TCAP-1 and restraint did not alter dendritic branching in the distal parts of the apical tree (Al Chawaf, 2008), although it increased spine density, increasing the number of spines relative to the saline-treated control. These results indicate that TCAP-1 did have effects on spine density in the apical tree, and the stratum radiatum was particularly affected. However, it is unknown whether these results translate to the CA1 region, as the effect of TCAP-1 on dendritic branching in the region is unknown. However, the stratum radiatum of the CA1 had significantly more spines than either the strata oriens or lacunsum-moleculare, and perhaps TCAP-1 had differential effects on this area as well.

In the amygdala, immobilization induces hypertrophy of dendritic arbour in BLA neurons (Mitra et al., 2005; Vyas et al., 2004, 2006), and an increase in dendritic spines (Vyas et al., 2006), however our findings indicate that daily restraint did not affect BLA spine density, and TCAP-1 had no effect in the BLA. It is possible that the daily restraint regimen used in these studies was not severe enough to cause morphological changes in amygdalar neurons although it significantly affected hippocampal neurons. This is possibly due to an insufficient induction of corticosterone (as discussed above), as only a single dose of physiologically high levels of corticosterone is sufficient to induce BLA hypertrophy and heightened anxiety (Mitra and Sapolsky, 2008).

Recent studies have indicated that individual differences to TCAP-1 may be important (Wang et al., 2005; Chapter 3), and therefore pooled results may confound significant data. The unstressed and restrained groups were analyzed together, and regression analyses indicated that there is a correlation between spine density in the CA3 and behaviours in the EPM (from Chapter 2). In restrained saline-treated animals, there was a significant positive correlation between spine density and open arm entries. This suggests that in saline-treated animals, higher spine density in the CA3 region is associated with greater entries into the open arm (and thus, decreased anxiety-like behaviour). As many stress manipulations, such as corticosterone treatment, result
in decreased spine density in the hippocampus (Morales-Medina et al., 2009) and increased anxiety-like behaviour in the EPM (Mitra and Sapolsky, 2008), one would surmise that increased spines result in a decrease in anxiety-like behaviour. However, the correlations with TCAP-1-treated animals indicate that the correlation is a negative relationship in restrained rats treated with TCAP-1, as higher spine density is associated with fewer entries into the open arm (and therefore, increased anxiety-like behaviour). Although one should expect that the relationship between spine number and anxiety-like behaviour should be independent of treatment, it is possible that with TCAP-1 treatment, TCAP-1 could have had an effect on “silent spines” (Bourne and Harris, 2008), or could be producing “aberrant spines” (Sunanda et al., 1995) which could result in increased anxiety-like behaviour in the EPM. To our knowledge, no studies have directly correlated spine density changes with anxiety-like behaviour in rats; although, a recent study in female rats reported a negative correlation between escape tendencies in a learned helplessness paradigm and hippocampal spine synapses (Hajszan et al., 2010). However, despite the high significance of the correlation in the current study, sample sizes are small and more anxiety tests must be used to properly delineate the role of individual differences on responses to TCAP-1.

This Chapter has shown that 10 days of TCAP-1 injection results in an increase in spine density in the hippocampus, but not the amygdala. While it appeared that these effects are lost under restraint conditions, as spine density in restrained TCAP-1-treated rats did not differ from restrained saline-treated rats, companion data describing the dendritic branching suggested that indeed, TCAP-1 may have increased total spine availability in the strata radiatum and lacunous-moleculare in the CA3 region. Finally, there were strong correlations between spine density and EPM behaviour (from Chapter 2), indicating that TCAP-1 might be altering anxiety-like behaviour, in part, via modification of dendritic spines.

4.6 References


Chapter Five: Uptake of intravenous TCAP-1 into body tissues and the brain

This chapter is in preparation for submission to Behavioural Brain Research:

Tan LA, Song L, Gifford A, Barsyte D, Lovejoy DA. Activity of subcutaneously-administered teneurin C-terminal associated peptide (TCAP)-1 in the blood and brain.

5.1 Abstract

Previous studies indicate that TCAP-1 is active in vivo at modulating anxiety-like behaviours under stress, and that TCAP-1 has promising neuroprotective effects under alkalotic (Trubiani, et al., 2007) or hypoxic stress (Ng, 2010). These results suggest that TCAP-1 could have therapeutic potential; however, in vivo TCAP-1 treatments have been mainly administered through the i.c.v. route, which is an invasive procedure. In contrast, one recent study showed that peripherally-administered TCAP-1 through the intravenous route modulated CRF-induced behaviour in the EPM and OF, and that a fluorescently-tagged TCAP-1 analogue could cross the blood-brain barrier (Al Chawaf et al., 2007). Previous results had indicated that TCAP-1 can be found in the limbic system, cortex, cerebellum, and hypothalamus of the rat brain, but it was unknown if these areas possessed binding sites for the peptide. Therefore, TCAP-1 was labelled with radioactive $^{125}$I and injected intravenously into rats. Organs and tissues were obtained and counted in a gamma counter, and the brain was sectioned to elucidate uptake sites in the brain. The results indicate that $[^{125}\text{I}]-\text{TCAP-1}$ is rapidly cleared from the blood via the gastrointestinal and renal tracts, and that only a small fraction of $[^{125}\text{I}]-\text{TCAP-1}$ enters the brain. Within the brain, $[^{125}\text{I}]-\text{TCAP-1}$ signal was found in areas of known TCAP-1 activity, such as the cortex, cerebellum, hippocampus, amygdala, and hypothalamus, but also in areas not known to have significant TCAP-1 expression such as the nucleus accumbens, substantia nigra, caudate putamen, superior and inferior colliculi, medial geniculate nucleus, and mammillary nucleus. The functions of these areas suggest that TCAP-1 may have additional roles in addiction, movement, memory, and the processing of auditory stimuli.

5.2 Introduction

The TCAP family consists of four novel neuropeptides that are found throughout the brain but also peripherally in the body (Chand et al., manuscript in preparation). Our laboratory has
demonstrated that *in vitro*, TCAP-1 is neuroprotective in immortalized neurons under alkaline (Trubiani et al., 2007) or hypoxic conditions (Ng, 2010). Previous chapters have also shown that TCAP-1 indeed has many *in vivo* effects that can affect behaviour, neuronal activation, and neuron morphology. However, to garner these *in vivo* effects, TCAP-1 was delivered in a large bolus directly into the lateral ventricles of rats, which is an undoubtedly invasive procedure. However, a previous study (Al Chawaf et al., 2007) indicated that TCAP-1 could modulate the effects of CRF when given peripherally. In addition, the study showed that a [K₈]-TCAP-1 analogue with a fluorescein isothiocyanate (FITC) tag could cross the blood-brain barrier (BBB) and could be found in blood vessels and fibres in the brain. Recent studies have indicated that TCAP-1 is rapidly cleared from the bloodstream after i.v. administration of the peptide and is undetectable a few hours after i.v. injection (Song, Barsyte and Lovejoy, manuscript in preparation). However, the mechanism by which TCAP-1 is eliminated is unknown.

Radioiodination is a method of labelling a peptide with a radionuclide such as ¹²⁵I or ¹³¹I, which are both γ-emitters. ¹²⁵I is the most common for radioiodination (Bailey, 1994) and ¹²⁵I can be attached to tyrosine residues in a peptide, making it amenable to localization in tissues by radioactive counts or localization in brain sections by visualization on radiographic film. Studies utilizing this technique allow researchers to detect low concentrations of peptide in brain sections.

Previous studies (Wang et al., 2005; Chand et al., manuscript in preparation) had determined that TCAP-1 is expressed and immunoreactive throughout the brain, especially in areas such as the hippocampus, hypothalamus, and cerebellum, and I have previously shown that TCAP-1 is active in reducing CRF-induced c-Fos (Chapter 3; Tan et al., 2009). However, the putative TCAP-1 receptor has not been found, and immunoreactive peptide labelling does not necessarily equate with peptide binding. Therefore, in collaboration with Dr. A. Gifford at InvivoPharm (Calverton, NY), TCAP-1 was iodinated and injected i.v. to determine uptake sites in the brain after [¹²⁵I]-TCAP-1 has crossed the BBB, as well as to determine the organs in the body that readily take up [¹²⁵I]-TCAP-1.
5.3 Materials and Methods

5.3.1 Animals

Male Sprague-Dawley rats (n = 5) (160 g, Charles River, Wilmington MA) were housed in standard laboratory conditions. All procedures were in accordance with the InvivoPharm company policies.

5.3.2 Peptides

Lyophilized TCAP-1 powder was provided to colleagues at InvivoPharm (Calverton, NY), where TCAP-1 radioiodination was completed by Dr. A. Gifford. 5 µl iodogen was added to 50 µl dichloromethane and allowed to dry. 30 µl of ammonium bicarbonate buffer was added, followed by 15 µg of TCAP-1. The solution was incubated with 1.5 mCi Na\(^{125}\)I in 40 µl phosphate buffer (pH 7.8) for 15 min at RT. The reaction was stopped with 2 µl trifluoroacetic acid (TFA) and purified by high-performance liquid chromatography (HPLC). A radioactive peak eluting between 13-15 min was collected and 870 µCi of radiolabelled peptide was obtained. \([^{125}\text{I}]\text{-TCAP-1}\) was stored in the mobile phase with 1% bovine serum albumen and 0.1% mercaptoethanol. Prior to injection, \([^{125}\text{I}]\text{-TCAP-1}\) was dried under nitrogen, re-dissolved in 0.1% TFA and loaded onto a C-18 sep pack cartridge. Following washing, the peptide was eluted with 50% acetonitrile with 0.1% TFA, evaporated to dryness and re-dissolved in 0.1 ml of ammonium bicarbonate and diluted in 0.5 ml ddH\(_2\)O.

5.3.3 Distribution of \([^{125}\text{I}]\text{-TCAP-1}\) in brain and tissues

To determine the distribution of \([^{125}\text{I}]\text{-TCAP-1}\) in rat tissues, Sprague-Dawley rats (n = 2) were first injected with 0.1 ml of intraperitoneal (i.p.) 0.9% sodium iodide prior to injection of radioiodinated TCAP-1 (10-12 µCi) via the tail vein. Rats were sacrificed 30 min after injection and tissues and organs were collected, weighed, and the radioactivity was counted in a gamma counter (CRC-5, Capintec Inc., Ramsey, NJ). Counts for the stomach, intestine, cecum, and colon included organ contents. Counts for the pancreas, prostate, skin, muscle, and bone were tissue samples rather than whole organs. To determine the distribution of \([^{125}\text{I}]\text{-TCAP-1}\) in the regions of the brain, Sprague-Dawley rats (n = 3) were weighed and injected with 0.2 ml of 0.9% sodium iodide (i.p.) prior to tail vein injection with \([^{125}\text{I}]\text{-TCAP-1}\). The dose (80-174 µCi) was proportional to the rat weight. Rats were sacrificed 30 min after \([^{125}\text{I}]\text{-TCAP-1}\) injection.
Following sacrifice, the brains were dissected out and weighed. Brains were cooled in ice-cold saline and sectioned in 300-µm slices on a vibratome. Sections were dried on a slide warmer and exposed to a phosphor screen for 7-15 days and scanned. Areas of $[^{125}\text{I}]-\text{TCAP-1}$ uptake in the brain were analyzed according to the atlas of Paxinos and Watson (1998).

5.4 Results

5.4.1 Uptake of intravenous TCAP-1 into body tissues

After i.v. injection, $[^{125}\text{I}]-\text{TCAP-1}$ could be found in the tissues throughout the rat body (Table 5.1) but tended to be associated with highly vascular tissues. The highest levels of $^{125}\text{I}$-TCAP-1 in terms of counts per minute (cpm) per gram of tissue included bladder, kidney, plasma stomach and liver. Lower concentrations were found in the adrenal gland, lungs, intestine and thyroid gland. The lowest concentrations were found in brain, cecum, muscle, testes and thymus. The bladder, when full, accounted for 2.7% of the total injected dose (ID), but when empty accounted for only 0.09% of the total ID. The kidneys took up 9.98% of the ID. A measurement of 5.39% of the ID of $^{125}\text{I}$-mTCAP-1 could also be found in the stomach and 6.41% could be found in the liver. The small intestine accounted for 4.59% of the ID. Lower activity was detected in the lungs, pancreas, spleen, cecum, adrenals, and skin. Only 0.13% of the total ID entered the brain.

5.4.2 Uptake of intravenous TCAP-1 into the brain

I.v. injection of $[^{125}\text{I}]-\text{TCAP-1}$ into the tail vein produced a distribution of radioactivity in the rat brain (Figure 5.1). Positive signals could be detected in the caudate putamen, nucleus accumbens (shell), thalamus, basolateral nucleus of the amygdala, hippocampus, substantia nigra, medial geniculate nucleus, mammillary nucleus, superior and inferior colliculus, and cerebellum. Positive but weaker signals could be detected in the preoptic area, hypothalamus, posteromedial cortical nucleus of the amygdala, and lateral entorhinal cortex. No signal could be found in the bed nucleus of the stria terminalis, septum, paraventricular nucleus of the hypothalamus, and locus coeruleus.
Table 5.1: Partitioning of $[^{125}\text{I}]$-TCAP-1 in tissues following i.v. administration to male rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean (cpm/g)</th>
<th>Range (cpm/g)</th>
<th>%ID/organ</th>
<th>% ID/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>50784</td>
<td>4580</td>
<td>0.05%</td>
<td>0.75%</td>
</tr>
<tr>
<td>Bladder (full)</td>
<td>902418</td>
<td>#</td>
<td>2.70%</td>
<td>12.29%</td>
</tr>
<tr>
<td>Bladder (empty)</td>
<td>76684</td>
<td>#</td>
<td>0.09%</td>
<td>1.21%</td>
</tr>
<tr>
<td>Bone</td>
<td>30582</td>
<td>1237</td>
<td>*</td>
<td>0.45%</td>
</tr>
<tr>
<td>Brain</td>
<td>5041</td>
<td>335</td>
<td>0.13%</td>
<td>0.07%</td>
</tr>
<tr>
<td>Cecum</td>
<td>9743</td>
<td>613</td>
<td>0.41%</td>
<td>0.14%</td>
</tr>
<tr>
<td>Colon</td>
<td>18865</td>
<td>2202</td>
<td>0.45%</td>
<td>0.28%</td>
</tr>
<tr>
<td>Heart</td>
<td>28366</td>
<td>185</td>
<td>0.25%</td>
<td>0.42%</td>
</tr>
<tr>
<td>Kidneys</td>
<td>414098</td>
<td>26374</td>
<td>9.98%</td>
<td>6.05%</td>
</tr>
<tr>
<td>Liver</td>
<td>75424</td>
<td>9691</td>
<td>6.41%</td>
<td>1.10%</td>
</tr>
<tr>
<td>Lungs</td>
<td>57749</td>
<td>1918</td>
<td>0.87%</td>
<td>0.85%</td>
</tr>
<tr>
<td>Muscle</td>
<td>18608</td>
<td>293</td>
<td>*</td>
<td>0.27%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>43809</td>
<td>3410</td>
<td>*</td>
<td>0.64%</td>
</tr>
<tr>
<td>Plasma</td>
<td>126250</td>
<td>1248</td>
<td>*</td>
<td>1.85%</td>
</tr>
<tr>
<td>Prostate</td>
<td>28861</td>
<td>4026</td>
<td>*</td>
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</tr>
<tr>
<td>Skin</td>
<td>36377</td>
<td>3770</td>
<td>*</td>
<td>0.53%</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>44427</td>
<td>162</td>
<td>4.59%</td>
<td>0.65%</td>
</tr>
<tr>
<td>Spleen</td>
<td>45698</td>
<td>1554</td>
<td>0.43%</td>
<td>0.67%</td>
</tr>
<tr>
<td>Stomach</td>
<td>122290</td>
<td>26368</td>
<td>5.39%</td>
<td>1.77%</td>
</tr>
<tr>
<td>Tail (injection site)</td>
<td>83638</td>
<td>47436</td>
<td>5.67%</td>
<td>1.28%</td>
</tr>
<tr>
<td>Testis</td>
<td>16403</td>
<td>1246</td>
<td>0.39%</td>
<td>0.24%</td>
</tr>
<tr>
<td>Thymus</td>
<td>18304</td>
<td>728</td>
<td>0.12%</td>
<td>0.27%</td>
</tr>
</tbody>
</table>

cpm/g = counts per minute per gram of tissue. %ID = percent radioactivity of injected dose. N = 2 for all tissues except for the bladder measurements where n = 1. * = full organs could not be obtained.
Figure 5.1: The distribution of intravenously injected $[^{125}\text{I}]-\text{TCAP-1}$ in the rat brain. Autoradiograms of 300 µm sections (left) with brain atlas reference (right; Paxinos and Watson, 2008) indicate areas of TCAP-1 binding. AcbSh, nucleus accumbens, shell; Amyg, amygdala; CB, cerebellum; CPu, caudate putamen; Hi, hippocampus; IC, inferior colliculus; mGN, medial geniculate nucleus; MN, mammillary nucleus; PAG, periaqueductal grey; SC, superior colliculus; SN, substantia nigra.
5.5 Discussion

TCAP-1 is a novel neuropeptide that is expressed and immunoreactive throughout the brain but is also found peripherally in areas such as the ovaries and testes (Chand et al., manuscript in preparation; Wang et al., 2005). However, areas of mRNA and peptide expression do not necessarily equate with peptide binding, and a membrane-bound receptor has yet to be conclusively identified. The work detailed in this chapter investigated the uptake sites of radioiodinated $^{125}$I-TCAP-1 both in the brain and in the body. I.v. injection of $^{125}$I-TCAP-1 resulted in uptake by tissues and organs of the body 30 min after administration. These studies indicate that i.v. TCAP-1 was preferentially taken up by regions in the limbic system, areas of high dopaminergic activity, and acoustic processing. Because the uptake was found in these particular regions and not necessarily those associated with high vascularity, this suggests a specific uptake mechanism for TCAP-1.

In the body, a large proportion of the labelled $^{125}$I-TCAP-1 was found in the kidneys and the full but not the empty bladder, indicating that exogenously-administered TCAP-1 is rapidly cleared via renal elimination. Large amounts of activity was also found in the liver, stomach, small intestine and its contents, indicating that TCAP-1 may be excreted through the gastrointestinal system. Although the gonads have been identified as an area of high TCAP-1 immunoreactivity (Chand et al., manuscript in preparation), the testes and prostate did not take up a notable amount of $^{125}$I-TCAP-1. However, low levels in the testes may be due to the protective effect of the blood-testis barrier (BTB), which acts like the BBB to prevent toxins from entering the seminiferous tubules (Mruk and Cheng, 2010). Interestingly, although $^{125}$I-TCAP-1 appears to cross the BBB, only a fraction of the dose at the 30 min time-point entered the brain. As low doses of i.v.-administered TCAP-1 have been shown to modulate anxiety-like behaviour (Al Chawaf et al., 2007), this supports previous results (Wang et al., 2005) that TCAP-1 is behaviourally active at very low doses.

The idea that a peptide could cross the BBB was once controversial; researchers had once believed the BBB to be almost impenetrable to larger molecules like peptides. However, the BBB is now seen as a regulatory system which allows some, but not all, peptides into the brain through transmembrane diffusion (TRH and α-MSH, for example) and carrier-mediated transport or saturable transport (AVP, leptin, and insulin, for example) (Banks et al., 1992). However,
peptides like melanin concentrating hormone (MCH), NPY, CART, orexin A, and orexin B cannot cross the BBB (Banks, 2006). BBB-crossing mechanisms involve proteins that allow transport into the brain; however, peptides that have similar or even the same receptors do not necessarily cross the BBB with equal efficiency. For example, the peptides MIF-1 and interleukin-1 cross the BBB but Tyr-MIF-1 and interleukin-2 do not (Kastin et al., 1999). CRF, which has a structure and molecular weight similar to TCAP-1, has an energy-dependent saturable transport out of the brain and a slow saturable influx into the brain (Kastin and Pan, 2003), but transport of peptides out of the brain may make the passage of peptides across the BBB to appear low. At the 30 min-timepoint, only 0.13% of TCAP-1 entered the brain. However, although peptide transport systems may exist to cross the BBB, this does not necessarily ensure that large amounts of peptide will enter the brain. The pancreatic peptides, amylin and insulin, require BBB transport mechanisms to access hypothalamic and cortical receptors, and the small amounts that cross the BBB are sufficient to reduce food intake and body weight. Only 0.12% and 0.046% of the injected dose of radiiodinated amylin and insulin, respectively, could be found in the brain (Banks and Kastin, 1998). While this may appear to be a very small proportion, in comparison, only 0.02% of a therapeutically active peripheral dose of morphine crosses the BBB (Kastin and Pan, 2003), and therefore, low concentrations in the brain can still have high efficacy. Other peripheral peptides, such as leptin, require BBB-crossing mechanisms to exert their effects. For example, leptin transport across the BBB is decreased in obese animals, and leptin administered to the brain, but not peripherally, caused the animals to lose weight (Banks, 2006). Thus, saturable influx mechanisms allow peripherally secreted peptides to enter the brain to exert its effects.

*In situ* hybridization, TCAP-1 immunohistochemistry, and c-Fos immunohistochemistry have indicated that the amygdala, medial prefrontal cortex, hypothalamus, cerebellum, and dorsal raphe nucleus are areas where TCAP-1 is either expressed or is active (Chand et al., manuscript in preparation; Tan et al., 2009; Wang et al., 2005). Using a FITC-[K8]-TCAP-1 analog, we previously showed that peripherally-administered TCAP-1 can cross the BBB. FITC-[K8]-TCAP-1 was found in several regions along large blood vessels, fibres, and highly vascularized areas such as the caudate putamen and choroid plexus, but not at any of the circumventricular organs (Al Chawaf et al., 2007). *In vitro* neuron cultures will readily take up FITC-[K8]-TCAP-1 in a punctate-like manner (Chand et al., manuscript in preparation), but the Al Chawaf et al.
(2007) study did not confirm uptake in many of the areas that previously showed TCAP-1 expression, and questions arose as to whether FITC-[K$_8$]-TCAP-1 was reaching the brain parenchyma.

Radioiodinated [$^{125}$I]-TCAP-1 was utilized as a different method to identify TCAP-1 binding sites in the brain. [$^{125}$I]-TCAP-1 was found, as expected, in areas previously known to have TCAP-1 expression, such as the hippocampus, amygdala, cortex, and cerebellum, but also in areas previously unknown to have significant TCAP-1 expression in the rat, such as the caudate putamen, nucleus accumbens, substantia nigra, mammillary nucleus, superior and inferior colliculi and medial geniculate nucleus.

Interestingly, there was a strong signal from the nucleus accumbens, substantia nigra, and caudate putamen, three areas of high dopaminergic activity. Although the resolution was not sufficient to detect a signal in the ventral tegmental area, TCAP-1 uptake in the nucleus accumbens suggests a role in reward systems (Leshner and Koob 1999; Self, 1998; Wise, 1987). Interestingly, recent data has indicated that TCAP-1 blocks CRF-induced cocaine reinstatement and behavioural sensitization (Kupferschmidt et al., 2011), although TCAP-1 had no effect on footshock- or cocaine-induced reinstatement or sensitization. The substantia nigra in the midbrain is the source of ascending dopaminergic connections to the dorsal striatum via the nigrostriatal pathway to control movement (Amalric and Koob, 1993). Uptake was also found in the caudate putamen in the rat, which suggests that TCAP-1 may have a role in elements of both the mesolimbic and nigrostriatal dopaminergic pathways and thus may have a role in motivation and addictions.

The mammillary nucleus (MN) is considered a part of the dorsal hypothalamus that has connections to the hippocampus and thalamic nuclei. The MN has been implicated for its role in memory (Vann and Aggleton, 2004) and the head-direction signal which helps encode the animal’s direction in the context of the environment (Taube, 2007). The nucleus is divided into the lateral and medial parts, which have different connections. The medial nucleus receives information from the septum, supramammillary nuclei, ventral tegmental area, entorhinal cortex, and the hippocampus, whereas the lateral nucleus receives input from the septum, supramammillary nuclei, dorsal tegmental nucleus, and the hippocampus, but it is assumed that the two nuclei are synergistic (Vann and Aggleton, 2004). Unfortunately, the resolution of the
autoradiogram did not allow for dissection of this nucleus, although in the capuchin monkey, TCAP-1 peptide has been found in the lateral but not medial MN (Casatti and Torres, personal communications). Lesions of the MN increase open arm entries in the EPM (Beracochea and Krazem, 1991), increase activity in the open field (Field et al., 1978), and impair memory in the radial-arm maze and the Morris water maze (Vann and Aggleton, 2004). These results are similar to those seen after hippocampal lesion (Bannerman et al., 2004) in which ventral lesions decrease anxiety-like behaviour and dorsal lesions impair spatial learning. Behaviourally, injections of TCAP-1 results in modulation of the stress response (see Chapter 2), and this suggests that TCAP-1 could have a role in spatial reasoning via uptake in this region.

The inferior colliculus (IC) is one of the largest midbrain structures that receives multimodal auditory information from the cochlear nuclei in the brainstem and sends ipsilateral and contralateral connections to the medial geniculate nucleus (mGN). Electrical stimulation of the IC elicits arousal, freezing, and escape behaviours in a dose-dependent manner in rats (Brandão et al., 2003), suggesting that the IC is intimately involved in the responses to a spectrum of stress severity. Moreover, injections of excitatory amino acids into the IC, enough to stimulate freezing behaviour, increase the amplitude of neuron potentials in the IC, similar to a response seen after the presentation of a conditioned stimuli (light) or ultrasonic signals (Brandão et al., 2001). It has been suggested that the IC, in addition to the dorsal periaqueductal grey, is responsible for the “brain aversion system” which controls defensive behaviours, such as vigilance, freezing, risk-assessment, and escape (Brandão et al., 2003). The mGN is a thalamic relay between the amygdala, thalamus, IC, and auditory cortex. The mGN also has indirect and direct connections to the amygdala, which are required for the conditioned fear response (LeDoux et al., 1990; LeDoux, 1993). Lesions of the mGN abolish HPA activation to noxious auditory stimuli, but not to restraint or ether (Campeau et al., 1997). Injections of TCAP-1 directly into the BLA modulated anxiety-like behaviour in the acoustic startle test (Wang et al., 2005) whereas i.c.v. injections of TCAP-1 decrease startle both in the absence (Wang et al., 2005) or presence of a stressor (Tan et al., 2008). The BLA is downstream of mGN input to modulate fear, suggesting that the decrease in the startle response may be a result of i.c.v. TCAP-1 reaching the mGN in addition to BLA modulation. Indeed, the BLA is required for synaptic plasticity in the mGN in response to auditory fear conditioning (Maren et al., 2001). The cells of the mGN are also sensitive to stressors, as chronic stressors have been shown to induce dendritic
atrophy in the mGN without altering auditory fear conditioning (Dagnino-Subiabre et al., 2009). That TCAP-1 appears to be taken up in both the IC and mGN suggests that TCAP-1 may be modulating both acoustic startle and defensive behaviours (see Chapter 2) via modulation of both these auditory inputs.

$^{125}\text{I}$-labelling of TCAP-1 is a less disruptive method of peptide labelling because of the comparatively small size of the iodine addition. This would be expected to have less of an effect on transport and binding in cells compared to a larger label such as FITC. This method also uses a native form of TCAP-1, and does not require substitution of lysine residues to attach the FITC tag. However, $^{125}\text{I}$ is an indirect method and we cannot be sure how much of the radioactive signal is associated with the nuclide per se or digested fragments of the peptide. On the other hand, the FITC label may provide more direct evidence of TCAP-1 localization because FITC by itself does not significantly label regions in the brain (Al Chawaf et al., 2007) and in vitro the FITC-labelled regions in cells show co-localization with immunoreactive peptide indicating that the peptide is intact (Chand et al., manuscript in preparation). FITC labels are also superior to radiiodination in that higher resolution images can be obtained from brain sections and no special radioactive protocols need be in place to perform the experiments. Additional studies using a comparable dose of i.c.v. FITC-$^{[K_8]}$-TCAP-1 will need to be completed, as the current study indicates that TCAP-1 does reach brain parenchyma.

Previous work has demonstrated that TCAP-1 has numerous effects in the brain; however, if a mechanism exists to transport peripheral TCAP-1 to the brain, this suggests that there must be some interaction between TCAP-1 in the periphery and TCAP-1 in the brain. At this time, it is unknown whether there is transport of TCAP-1 out of the brain into the blood, like the saturable system that exists for CRF. In addition to its numerous effects in the brain, CRF has important stress-related effects in the periphery. CRF receptors are richly expressed in the gastrointestinal tract (Larauche et al., 2009) and mediate gut motility during stress. In fact, the CRF$_1$ receptor has been implicated in gastrointestinal diseases such as irritable bowel syndrome. Therefore, TCAP-1 could also have peripheral effects in body tissues, perhaps separate from its effects in the brain; however, it is unknown whether the gastrointestinal tract expresses TCAP-1. There is high TCAP-1 immunoreactivity in the testes and ovaries, particularly in cell types that have high proliferative rates, such as the spermatagonia and spermatocytes in the testes, and the granulosa cells and oocytes of the ovaries (Chand et al., manuscript in preparation). Stress mediators, such
as CRF and glucocorticoids, interfere with tissues secreting sex steroids (Rivier and Rivest, 1991), inhibiting reproduction during periods of stress. TCAP-1 may therefore be interacting with the hypothalamic-pituitary-gonadal (HPG) axis, modulating the effects of these stress mediators at the level of the gonads but also feeding back and affecting centres in the brain governing reproduction. Alternatively, TCAP-1 release from the gonads to the brain may mediate stress sensitivity during receptive periods to affect reproductive behaviours.

In summary, the data detailed in this chapter indicates that peripherally-administered $[^{125}\text{I}]$-TCAP-1 enters the bloodstream and is most likely rapidly eliminated via the renal and gastrointestinal systems. Like other bioactive peptides that pass through the BBB, only a small fraction of the peptide entered the brain. There, $[^{125}\text{I}]$-TCAP-1 was taken up by structures in the limbic system as well as elements of the dopaminergic system and auditory processing relays.

5.6 References


6  Chapter Six: Significance of findings and conclusions

6.1  Abstract

The studies completed in this thesis have advanced the knowledge of TCAP-1 \textit{in vivo}. I determined that intracerebral TCAP-1 can modulate anxiety-like behaviours in rodent exploratory tests of anxiety, depending on the level of stress perceived by the rat. Next, I elucidated the areas in the brain where TCAP-1 binds and is active in blocking CRF-induced neuronal activation, such as within the limbic system and brainstem nuclei. Finally, I determined that administration of TCAP-1 can remodel dendritic spines in the hippocampus, an important stress-sensitive and regulatory centre of the HPA axis, without affecting the amygdala. Although TCAP-1 has structural similarity to the CRF family of peptides, TCAP-1 itself may not induce a stress response, instead lowering the threshold of the stimulus required to initiate stress behaviours, resulting in what may seem like paradoxical behaviour. Furthermore, the results of this thesis provide a set of clear questions to further investigate the role of TCAP-1 in stress, memory, and behavioural adaptation. Possible experimental approaches have been proposed that could tackle these new questions.

6.2  The neuroanatomy of TCAP-1

The neuroanatomy involved in the stress response is understandably a very complex network of interconnected nuclei that activate, inhibit, and disinhibit both the endocrine and autonomic outputs of the brain to integrate sensory information and mount the correct response to harmful stimuli. CRF, glucocorticoids, and monoamines all participate in this integration, but a new player, namely TCAP-1, provides another level of endogenous control on specific stress-regulating nodes in the brain.
Figure 6.1: Neuroanatomy of TCAP-1 within stress circuitry. TCAP-1 is acutely active in areas of the limbic system and the brainstem (yellow arrows) where it decreases the activation of stress-sensitive nuclei that have direct or indirect connections to the PVN. GABAergic (red lines) and glutamatergic (green lines) input into PVN-projecting areas, such as the BnST and peri-PVN, which themselves are GABAergic, modulate input from the limbic areas. Monoaminergic input from the brainstem (grey arrows) project to both PVN-projecting areas as well as the mPFC, but its CRF-induced activation from the PVN may be blunted by TCAP-1. Adapted from Tan and Lovejoy, 2009.
Based on the studies completed in this research, along with emerging new evidence on the mechanism of TCAP-1, I propose a mechanism for the actions of TCAP-1. Once peripheral TCAP-1 has crossed the BBB, or has been released in the brain, TCAP-1 is taken up by the hippocampus, amygdala, cortex, and dopaminergic and serotonergic nuclei. Under normal conditions, TCAP-1 increases spine density in the hippocampus but not the amygdala, although it does not activate c-Fos-mediated processes. However, during times of stress, such as during the release of CRF in the brain, which mimics the onset of a stress-like episode, TCAP-1 blocks the effect of CRF in the mPFC, hippocampus, amygdala, and dorsal raphe (Figure 6.1). This may result in a decrease of the glutamatergic input from the mPFC and hippocampus to areas such as the BnST and peri-PVN, which send GABAergic connections to the PVN. Additionally, TCAP-1 may decrease GABAergic input from the amygdala to the BnST and peri-PVN area and serotonergic input from the dorsal raphe to the peri-PVN area, perhaps through activation of a different set of neurons in these relay areas than those innervated by inhibitory inputs. As TCAP-1 administration did not decrease activation of the BnST as a whole, it is possible that decreased glutamatergic input to the BnST from the mPFC and hippocampus compensated for a decrease in GABAergic input from the amygdala, resulting in unabated PVN activation. But because each of these brain areas is involved in different aspects of the behavioural stress response, TCAP-1 may increase the sensitivity to stimuli which would alter behavioural, rather than HPA axis output. Although the neuroanatomy of TCAP-1 suggests a role in glutamate and GABA modulation, it was not determined directly as a part of my work. Previous studies indicate that TCAP-1 can modulate the actions of glutamate on immortalized hypothalamic cells (Trubiani, 2008). However, further studies will need to be performed to confirm such a mechanism.

6.3 TCAP-1: An enigmatic peptide

During the time in which my thesis research was performed, a set of recent data has become available that helps conceptualize the conditions under which TCAP-1 can be released. TCAP-1 can be expressed as a separate transcript from the teneurin transcript, as cDNA probes directed towards the TCAP-1 sequence revealed two separate bands in a Northern blot, representing a full-length teneurin transcript and a shorter TCAP transcript roughly the size of the terminal exon (Chand et al., manuscript in preparation). This suggests that although the TCAPs have been
associated with the teneurins, TCAP-1 could be independently transcribed from the TCAP sequence that is associated with the teneurins. Recent immunohistochemistry data support this theory. E14 immortalized hippocampal cells were labelled with antibodies generated against epitopes within the TCAP-1 sequence, and compared with labelling from antibodies for teneurin just upstream of the TCAP-1 sequence; whereas the TCAP-1 antiserum predominantly labelled the cytosol, the teneurin antiserum labelled the plasma membrane. Although there was some overlap, there were areas of distinct TCAP-1-only signals (Chand et al., manuscript in preparation). Endogenous TCAP-1 does not appear to be sequestered in vesicles, and it is possible that TCAP-1 is transcribed by free ribosomes, which is common in peptides that are soluble in the cytosol, such as fibroblast growth factor (FGF)-1 (Jackson et al., 1992). Thus, a reservoir of cytosolic TCAP-1 exists in these cells expressing endogenous TCAP-1. Addition of fluorescently-labelled FITC-[K₈]-TCAP-1 to either E14 or primary hippocampal cells shows that FITC-[K₈]-TCAP-1 is readily taken up by neurons in a punctate-like manner, and in some cases, the FITC signal can be found in the nucleus (Chand et al., manuscript in preparation). The mechanism for this uptake has not been elucidated as yet, although it is not clathrin-dependent (Chand and Song, unpublished findings).

Before the evolution of multicellular organisms and circulatory systems, one of the simplest forms of communication between cells was the paracrine release of molecules into the media in which the cells were living, which would reach the target cells via diffusion. These chemical signals could be released under a variety of conditions, including upon necrotic cell death, when the cellular membrane would degenerate and the cytosolic contents would be released into the surrounding area. Recent data has indicated that TCAP-1 has neuroprotective effects under both alkalotic and hypoxic conditions. Under conditions of high pH, TCAP-1 upregulated superoxide dismutase-1 as well as the copper chaperone, inhibiting necrosis in the N38 neuronal cell line (Trubiani et al., 2007). Additionally, TCAP-1 increased cell proliferation of N38 cells under 1% oxygen relative to vehicle-treated controls (Ng, 2010). Thus, TCAP-1 could be acting upon neighbouring cells as a paracrine signal that cells around it were perishing so as to upregulate survival pathways in order to survive (Figure 6.2).
Figure 6.2: Model of TCAP-1’s intracellular effects. Exogenous TCAP-1 binds to a membrane-bound receptor and is taken up in a punctate-like manner. Exogenous TCAP-1 upregulates neuroprotective mechanisms, such as superoxide dismutase (SOD), SOD copper chaperone (SDCC), and catalase, and the modulation of BDNF expression and release. TCAP-1 upregulates α-actinin and β-tubulin, which increases cytoskeletal proteins. Endogenous TCAP-1 in the cytosol binds to elongation factor-1 (EF-1), which helps anchor actin mRNA to the cytoskeleton to produce new dendritic spines. Adapted from Tan and Lovejoy, 2009.
These signals could also have major effects on the cytoskeleton, stimulating the neurons in a network to prepare for a decrease in number, thus preparing new connections to compensate for a loss of connectivity. TCAP-1 affected neurite outgrowth in N38 cultures, modulating neurite number under normal (Al Chawaf et al., 2007a) and hypoxic conditions (Ng, 2010). Furthermore, TCAP-1 modulated neurite length, increasing the length of long neurites and decreasing the length of short neurites (Al Chawaf et al., 2007a). It appears that TCAP-1 is vital for this reorganization of neurites in the neuron. Knockdown of all four TCAPs using siRNA resulted in a decrease in neurite number (Trubiani, 2008). These effects could only be accomplished via remodelling of the neuronal cytoskeleton. TCAP-1 increased β-actin and β-tubulin cytoskeletal proteins and increased α-actinin-4 and β-tubulin transcription in vitro (Al Chawaf et al., 2007a). Interestingly, administration of TCAP-1 on E14 hippocampal neurons caused a reorganization of β-tubulin-immunoreactive fibres from a cytosolic scaffold to the periphery of the cell (Chand and Lovejoy, personal communications). This indicates that TCAP-1, in addition to protecting the cell from a potential insult, could be promoting active mechanisms to maintain network integrity.

6.4 TCAP-1 and CRF: Preparing the brain for stress

In the brain, the perception of a stressor, whether real or imagined, produces a cascade of signals throughout autonomic and neuroendocrine pathways to prepare the body for the insult. Increasing the concentration of a neuroprotective peptide could therefore modify the activity of these systems to modulate the effect of the incoming stimuli. CRF is released both in the PVN to the HPA axis as well as within central brain circuits like the limbic system and brainstem. Acute TCAP-1 on its own, although it had no effects on c-Fos protein, blocked CRF-induced c-Fos immunoreactivity in several regulatory areas that project to the PVN and hence the HPA axis (Chapter 3). However, TCAP-1 did not affect recruitment of the PVN neurons, suggesting that TCAP-1 could act independently of the HPA axis. Previously, i.v. TCAP-1 did not affect i.v. CRF-induced corticosterone release (Al Chawaf et al., 2007b), indicating that TCAP-1 does not interfere with CRF₁ receptors in the pituitary. On the other hand, central CRF₁ receptors may have different responsibilities that are independent of HPA axis, as conditional knockout of forebrain CRF₁ receptors decreased anxiety-like behaviour independently of the HPA axis.
Therefore, TCAP-1 may be blocking CRF$_1$ receptor activity without affecting HPA axis recruitment.

In contrast to acute administration, repeated administration of TCAP-1 in a pre-treatment protocol did not affect CRF-induced c-Fos immunoreactivity (Chapter 3). C-Fos protein has low basal expression, increases rapidly after stimuli, and returns to basal levels 4 hours after an insult (Bittencourt and Sawchenko, 2000). Therefore, TCAP-1’s effects on c-Fos may only extend to this short window, and not one week after TCAP-1 administration, instead producing long-term effects of TCAP-1 administration downstream of the c-Fos pathway. Acute and repeated administration of TCAP-1 could have different effects on CRF-induced c-Fos. In both the acute and repeated treatments, TCAP-1 increased the variability in the group treated with both TCAP-1 and CRF. This suggests that there may be individual differences in the responses of rats to TCAP-1 under stress; some rats had consistently high c-Fos levels, whereas others had consistently low c-Fos levels. TCAP-1 could be modulating stress responses in the brain, polarizing the responses to either potentiate or attenuate a response, depending on the rat.

Over time, repeated exposure to mediators of stress produces profound effects on the brain. Chronic exposure to corticosterone remolds neurons in the hippocampus (Morales-Medina et al., 2009) but exposure to mild stressors, such as our model of repeated restraint, may induce compensatory measures to maintain connectivity. Prolonged exposure to a peptide released during cell death could prepare the brain for additional insults by increasing the connections in a network. Repeated administration of TCAP-1 increased spine density in the hippocampus without affecting the amygdala (Chapter 4). Prolonged exposure to stressors also affected spine density, as repeated restraint appeared to increase spine density with or without exogenous TCAP-1. In these rats, restraint treatment decreased dendritic branching relative to non-restrained rats in the apical tree, whereas TCAP-1 increased branching in the apical tree in restrained rats relative to saline-treated restrained rats (Al Chawaf, 2008). Total spine density changes as a function of dendritic length and an increase of spines may compensate for decreased dendritic branching (Radley and Morrison, 2005). Therefore, in saline-treated and restrained rats, spine density in the apical tree would effectively remain the same, whereas in TCAP-1-treated and restrained rats, spine density in the apical tree would be increased. The mechanism for increased spine density remains elusive (Figure 6.2). As mentioned previously, TCAP-1 remolds the cytoskeleton, and in addition, recent evidence has suggested that TCAP-1...
can bind to elongation factor-1 (Chand and Song, personal communications), which helps anchor β-actin mRNA to the cytoskeleton (Liu et al., 2002) and is found in the apical dendrites of the hippocampus (Huang et al., 2005). CRF is released from the interneurons of the hippocampus (Chen et al., 2001; 2004a), where it is well positioned to be released after stressful stimuli, and knockout of the CRF₁ receptor results in increased dendritic arborisation (Chen et al., 2004b). Therefore, blockade of CRF by TCAP-1 administration (Chapter 3), at least acutely, could account for increased branching in the hippocampus.

6.5 TCAP-1 in stress behaviours: My spider sense is tingling

The ability to actively perceive and cope with potentially harmful situations is no doubt beneficial for survival. In a new environment, animals are constantly faced with approach-avoidance conflict: whether to explore the new environment and gain valuable information or remain protected but ignorant to its surroundings. The stress state of the animal may play a factor. For example, in a new environment, low levels of CRF will be behaviourally activating, increasing exploration; whereas under high levels, CRF will inhibit exploration (Dunn and Berridge, 1990). A peptide that prepares the brain for stressors could therefore promote different coping mechanisms by increasing the exploratory drive in a novel environment (active coping) and increasing protective and defensive behaviours (passive coping) in a particularly harmful environment.

In the absence of a stressor, TCAP-1 appeared to have mild anxiolytic effects in the elevated plus maze (EPM) and open field (OF) (Chapter 2). TCAP-1 appeared to increase directed exploratory behaviour and increased open arm exploration when TCAP-1 was given repeatedly. As the open spaces in the EPM and OF induce a stress response, TCAP-1 could have lowered the stimulus threshold necessary to initiate active coping behaviours, enhancing the effects of a low-stress environment that culminated in increased exploratory behaviour. Although acute TCAP-1 on its own did not affect c-Fos expression (Chapter 3), it is possible that TCAP-1 may have blocked the effects of CRF released in response to the behavioural test, as the open spaces can produce an HPA axis response (Pellow et al., 1985).

In the presence of a stressor, TCAP-1 appeared to have anxiogenic effects in the EPM and OF tests. Acutely, TCAP-1 had no effect on CRF-induced behaviours, although TCAP-1 attenuated CRF-activation of the limbic system (Chapter 3). However, repeated exposure to TCAP-1
promoted stress-like behaviours. Five days of TCAP-1 pre-treatment or 10 days of repeated TCAP-1 treatment produced behaviours where rats avoided open spaces and decreased exploratory behaviour. As TCAP-1 release in the brain may signal brain distress or injury, the effects of repeated TCAP-1 may be compounded. This increase in anxiety-like behaviour may be adaptive over time, as continued exposure to a peptide that elicits a “prepared state”, including increased hippocampal connectivity (Chapter 4), but not necessarily a “stressed” state, could make an organism more aware of injurious cues. Instead, TCAP-1 may lower the threshold to initiate passive coping behaviours in the presence of a stressor, such as decreased exploration and xenophobia.

Previous studies have shown that TCAP-1 can have modulatory effects on stress behaviour. I.v. administration of TCAP-1 modulated CRF-induced behaviours in the EPM and OF, depending on the route of administration of CRF. TCAP-1 potentiated the effects of i.v. CRF but attenuated the effects of i.c.v. CRF. I.v. CRF does not appreciably cross the blood-brain barrier (BBB; Chrousos et al., 1985), although a saturable transport system across the BBB exists (Kastin and Pan, 2003). On the other hand, TCAP-1 does cross the BBB (Chapter 5; Al Chawaf et al., 2007b), but in very small quantities. I.v. TCAP-1 did not alter HPA axis induction (Al Chawaf et al., 2007b), and i.v. CRF mainly affects the pituitary CRF_1 receptors, suggesting that behavioural effects may have been the result of the central effects of TCAP-1. However, the dose of TCAP-1 that reached the brain is considerably lower than concentrations that were delivered i.c.v. in the above experiments. As TCAP-1 appears to exert its pharmacological effects via a dose-dependent inverse-U shaped curve (Wang et al., 2005), low central concentrations of TCAP-1 may have different effects than the higher doses seen in the i.c.v. TCAP-1 experiments.

In the acoustic startle test, two studies demonstrated that i.c.v. TCAP-1 has anxiety-reducing effects. On its own, pre-treatment of TCAP-1 for 5 days decreased average startle both in the absence (Wang et al., 2005) and presence of CRF (Tan et al., 2008). In response to noxious stimuli such as loud noise, like the results from the EPM and OF, TCAP-1 could have promoted passive coping styles, such as freezing and decreased reactivity to the environment (Bandler et al., 2000). This passive coping behaviour, which can occur in response to stressors that are inescapable, may culminate in decreased startle. Thus, TCAP-1 may be enhancing the switch between coping styles from active to passive depending on the level of stress, hence driving the
organism towards what may seem like paradoxical behaviour in these behavioural tests. This effect may be mediated by the trafficking of CRF receptors, as TCAP-1 may have an effect on the proportion of CRF₁ and CRF₂ receptors at the cell membrane. For example, in the serotonergic neurons of the dorsal raphe nucleus, exposure to stress induces an internalization of CRF₁ receptors from the membrane to the cytosol and the trafficking of CRF₂ receptors from the cytosol to the membrane (Waselus et al., 2009). As the CRF₁ receptor activation generally promotes active coping behaviours, and CRF₂ receptor activation generally promotes passive coping behaviours, altered trafficking of the CRF receptors may account for enhancements of both active and passive coping styles.

6.6 Stress as a risk factor for disease: Too much of a good thing

The stress response is essential for the survivability of an organism, and hence its genes, and is understandably a complex and deeply regulated system. The ability of an organism to cope with perturbations to homeostasis put it at a considerable advantage over those that cannot and therefore endogenous systems that mediate allostatic mechanisms, or “stability through change” (McEwen, 2008), are retained and conserved across species. However, continued exposure to even sub-threshold but damaging stimuli can overload a taxed system (“allostatic overload”). We see the consequences of this in the human population: the inability to deal with chronic stressors is a risk factor for mental, neurodegenerative, cardiovascular, and gastrointestinal diseases (McEwen, 2008). A prolonged or exaggerated response to stress causes damage to the brain, which may be a factor in the development of brain diseases. For example, chronic stress can induce hippocampal atrophy and a decrease in neurogenesis (Bremner, 2006; McEwen and Magariños, 1997), whereas small hippocampal sizes have been correlated with major depressive disorder and posttraumatic stress disorder (Bonne et al., 2008; Campbell et al., 2004). Furthermore, chronic activation of the CRF₁ receptor increases phosphorylation of tau proteins, a prerequisite for the formation of β-amyloid plaques that are prevalent in Alzheimer’s disease pathology (Rissman et al., 2007). Therefore, understanding the mechanisms that change the stress response from adaptive to maladaptive is vital to discovering new therapies, and can be accomplished by investigating the component parts of the stress response.

The TCAP family provides a new thread in the network of endogenous stress mediators. Although many questions remain as to how endogenous TCAP behaves, it is becoming clear that
TCAP’s role in the brain is an important one. Its influence may extend beyond the stress response to development, metabolism, and reproduction, and can therefore be a novel therapeutic target for numerous economically significant diseases.

6.7 Future directions

Throughout this thesis I have highlighted many of the findings that have advanced the knowledge of TCAP-1 in vivo. However, many questions remain as to how TCAP-1 may seemingly polarize the behavioural response to stress, and as to what other effects TCAP-1 has on stress behaviours, such as learning and memory.

6.7.1 How do the effects of endogenous TCAP-1 differ from synthetic TCAP-1?

Much of the early work on TCAP-1 has centred on the use of the synthetic peptide in both in vitro and in vivo experiments. However, as can be seen in experiments with i.c.v. Ucn 1, central injection can affect the whole brain, not only places where the endogenous peptide is released (Jones et al., 1998; Spiga et al., 2006). Therefore, i.c.v. TCAP-1 may be acting on sites where TCAP-1 is not normally found or released. Although the administration of TCAP-1 will no doubt be an important strategy in determining its role of in regulating CRF- and stress-related behaviours, deciphering the role of endogenous TCAP-1 in the brain will also be required. TCAP-1 antisera and 35S-TCAP-1 antisense probes have been successful in locating TCAP-1 distribution in the brain; however, these techniques can also be used to detect changes in TCAP-1 immunoreactivity or TCAP-1 mRNA expression in response to psychological or physical stressors. Additionally, these experiments may reveal the conditions upon which endogenous TCAP-1 is released in the brain.

The advent of new TCAP-1 over-expressing immortalized cells (Barsyte-Lovejoy and Lovejoy, personal communications) as well as TCAP-1 siRNA (Trubiani, 2008) will enable us to discern the role of endogenous TCAP-1 in mediating intracellular processes, whereas TCAP-1 siRNA could be microinfused into the hippocampus, amygdala, septum, or BnST to determine if endogenous TCAP-1 is mediating stress behaviour via the limbic system.
6.7.2 Does TCAP-1 affect HPA axis regulation?

Systemic TCAP-1 does not appear to affect HPA axis activation (Al Chawaf et al., 2007b), although this does not discount the possibility that higher concentrations of central TCAP-1 may affect HPA axis output by affecting PVN-regulating areas such as the mPFC, hippocampus, or amygdala. Therefore, it will be important to determine whether acute or repeated i.c.v. injections of TCAP-1 can alter HPA axis output (ACTH or corticosterone), and whether central TCAP-1 can block HPA axis recruitment in response to psychological stimuli, such as restraint or social defeat, that utilize forebrain processing before its relay to the PVN.

TCAP-1 appears to be most effective under multiple administrations of the peptide. Additional strategies such as longer dose regimens may enhance the effects seen in my experiments. Another possibility is the use of osmotic minipumps, usually used to continually administer a drug over time. TCAP-1 has a short half-life in serum, and daily i.c.v. injections expose the organism to daily peaks of high TCAP-1 concentration. Administration by minipump would facilitate a steady concentration of TCAP-1 for the duration of the regimen. This strategy may simulate an increased basal level of release rather than intermittent release modelled by daily injection.

Multiple administrations of TCAP-1 have produced the strongest behavioural effects. These effects last long after the peptide has been cleared from the bloodstream, indicating that long-term modifications have taken place. Whereas this could have occurred via remodelling of hippocampal dendritic spines, it is possible that TCAP-1 administration could have affected expression of a variety of receptors, including CRF$_1$, CRF$_2$, GR, or MR. Although TCAP-1 does not bind to CRF receptors (Nock, 2009; Wang et al., 2005), TCAP-1 could alter the expression of these receptors; for example, the expression patterns of CRF$_1$ receptors and immunoreactivity patterns of TCAP-1 overlap in areas such as the cortex, cerebellum, hippocampus, BLA, and pontine grey. TCAP-1 could upregulate CRF$_1$ receptors in these areas under conditions of TCAP-1 release (for example, an ischemic insult in which there is neural trauma), modulating a stress response, depending on the area in which CRF$_1$ receptor is upregulated.

The GR:MR ratio has been implicated in regulating stress responsiveness and adaptation (Oitzl et al., 1997). Whereas the GR is important in long-term memory processes, the MR appears to be implicated in short-term processes and novel behaviour (Oitzl et al., 1997). A higher GR:MR
ratio could result in increased responsiveness to stressors, whereas a lower GR:MR ratio could result in increased resilience and neuroprotection (Joëls and de Kloet, 1994). As the hippocampus highly expresses both GR and MR, it is the ideal place to investigate whether endogenous TCAP-1 and these receptors co-localize, and whether the GR:MR ratio in hippocampal cells changes in response to TCAP-1 administration.

6.7.3 Does TCAP-1 have a role in learning and memory?

Recent immunohistochemical work in both rat and monkey has supported c-Fos data indicating that the hippocampus is an important site for TCAP-1 activity. In the rat, TCAP-1 immunoreactivity is widespread in the hippocampus, with strong signals in the CA3 and CA2, a moderate signal in the CA1 and a weaker signal in the DG (Chand et al., manuscript in preparation). Interestingly, TCAP-1 antisera labelled dendritic branches in the apical tree of the CA1-CA3 subfields of the hippocampus (Casatti and Torres, personal communications) where the effects of TCAP-1 on increasing spine density were their greatest, with little immunoreactivity in the basilar tree (Figure 6.3). In the capuchin monkey (Cebus apella), TCAP-1 immunoreactivity is also found in the apical fibres of the hippocampal pyramidal neurons (Casatti and Torres, personal communications), with no immunoreactivity in the basilar tree.

The hippocampus has an established role in inhibiting the HPA axis and a role in stress behaviours, but perhaps its strongest influence is in spatial reasoning, learning, and memory. TCAP-1’s strong presence in the hippocampus, especially in the dorsal hippocampus, indicates that TCAP-1 could have a role in the processing and retention of salient information, perhaps promoting behavioural adaptation after continued exposure to a stimulus. Future work could utilize a variety of hippocampal-dependent rodent memory tasks, such as the 12-arm radial maze or the Morris water maze (Sharma et al., 2010) in which intrahippocampal administration of synthetic TCAP-1 or TCAP-1 siRNA could be used to investigate the roles of high TCAP-1 and low TCAP-1 on spatial learning.

Other types of learning, such as fear conditioning (Pitts et al., 2009) are dependent upon the CeA for memory consolidation but not for the expression of fear. The CeA contains CRF-immunoreactive cell bodies and fibres but does not express CRF receptors. As TCAP-1 attenuated the activation of the CeA, future studies could investigate whether microinjections of
TCAP-1 could interfere with consolidation of fear memories, uncoupling the relationship between the conditioned and unconditioned stimuli.

Figure 6.3: Photomicrographs of TCAP-1 immunoreactivity in hippocampal neurons from rat and capuchin monkey (*Cebus apella*). Left: Rat brain labelled with TNR408 antisera (1:1700) with nickel-enhanced DAB (magnification = 400x). Note that TCAP is found in the pyramidal layer (Py), stratum lucidum (SL), stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) but not the stratum oriens (SO). Right: Monkey brain labelled with TNR408 antisera (1:1700) with nickel-enhanced DAB (magnification = 160x). Note the absence of pyramidal soma labelling in the Py layer, but positive labelling in the dendritic fibres of the SR and SLM. Photomicrographs are courtesy of Prof. Claudio Casatti and Kelly Torres, Universidade Estadual Paulista (UNESP), Brazil.

6.8 Conclusions

In closing, these investigations have shown that TCAP-1, a newly-elucidated, endogenous, and highly-conserved peptide, has numerous *in vivo* effects that build upon recent *in vitro* data. My studies have confirmed a role for TCAP-1 in stress behaviours and brain plasticity, but the results suggest further roles in learning and memory. TCAP-1 could indeed be an allostatic mediator, limiting the allostatic overload that results in damage to the brain, and increasing protective behavioural strategies to ensure survivability. Thus, the data presented here lay a foundation of work that have both furthered our understanding of this enigmatic peptide and posed questions as to the nature of its irreplaceable functions in the body.
6.9 References


